

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁵ : C07K 7/06, 7/08, 7/10, A61K 37/02	A1	(11) International Publication Number: WO 94/20532 (43) International Publication Date: 15 September 1994 (15.09.94)
(21) International Application Number: PCT/US94/02465 (22) International Filing Date: 11 March 1994 (11.03.94) (30) Priority Data: 08/030,644 12 March 1993 (12.03.93) US 08/093,202 15 July 1993 (15.07.93) US 08/183,222 14 January 1994 (14.01.94) US (71) Applicant: XOMA CORPORATION [US/US]; 2910 Seventh Street, Berkeley, CA 94710 (US). (72) Inventor: LITTLE, Roger, G., II; 620 Rose Drive, Benicia, CA 94510 (US). (74) Agent: NOONAN, Kevin, E.; Allegretti & Witcoff, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: BIOLOGICALLY ACTIVE PEPTIDES FROM FUNCTIONAL DOMAINS OF BACTERICIDAL/PERMEABILITY-INCREASING PROTEIN AND USES THEREOF		
(57) Abstract <p>The present invention provides peptides having an amino acid sequence that is the amino acid sequence of a human bactericidal/permeability-increasing protein (BPI) functional domain or a subsequence thereof, and variants of the sequence or subsequence thereof, having at least one of the BPI biological activities, such as heparin binding, heparin neutralization, LPS binding, LPS neutralization or bactericidal activity. The invention provides peptides and pharmaceutical compositions of such peptides for a variety of therapeutic uses.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	MG	Madagascar	TJ	Tajikistan
DE	Germany	ML	Mali	TT	Trinidad and Tobago
DK	Denmark	MN	Mongolia	UA	Ukraine
ES	Spain			US	United States of America
FI	Finland			UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Biologically Active Peptides from Functional Domains of Bactericidal/ Permeability-Increasing Protein and Uses Thereof

5 This is a continuation-in-part of U.S. Patent Application 08/183,222
filed January 14, 1994, which is a continuation-in-part of U.S. Patent
Application 08/093,202, filed July 15, 1993, which is a continuation-in-part of
U.S. Patent Application Serial No. 08/030,644 filed March 12, 1993.

BACKGROUND OF THE INVENTION

10

The present invention relates to peptides derived from or based on
bactericidal/permeability-increasing protein and therapeutic uses of such
peptides.

15 Bactericidal/permeability-increasing protein (BPI) is a protein isolated
from the granules of mammalian polymorphonuclear neutrophils (PMNs), which
are blood cells essential in defending a mammal against invading
microorganisms. Human BPI has been isolated from PMNs by acid extraction
combined with either ion exchange chromatography (Elsbach, 1979, *J. Biol.*
Chem. 254: 11000) or *E. coli* affinity chromatography (Weiss *et al.*, 1987,
20 *Blood* 69: 652), and has potent bactericidal activity against a broad spectrum
of Gram-negative bacteria. The molecular weight of human BPI is
approximately 55,000 daltons (55kD). The complete amino acid sequence of
human BPI, as well as the nucleotide sequence of DNA encoding BPI, have
been elucidated by Gray *et al.*, 1989, *J. Biol. Chem.* 264: 9505, incorporated
25 herein by reference (*see* Figure 1 in Gray *et al.*).

The bactericidal effect of BPI has been shown to be highly specific to
sensitive Gram-negative species. The precise mechanism by which BPI kills
Gram-negative bacteria is not yet known, but it is known that BPI must first
attach to the surface of susceptible Gram-negative bacteria. This initial binding
30 of BPI to the bacteria involves electrostatic interactions between BPI, which is
a basic (i.e., positively charged) protein, and negatively charged sites on
lipopolysaccharides (LPS). LPS is also known as "endotoxin" because of the

potent inflammatory response that it stimulates. LPS induces the release of mediators by host inflammatory cells which may ultimately result in irreversible endotoxic shock. BPI binds to Lipid A, the most toxic and most biologically active component of LPS.

5 BPI is also capable of neutralizing the endotoxic properties of LPS to which it binds. Because of its Gram-negative bactericidal properties and its ability to bind to and neutralize LPS, BPI can be utilized for the treatment of mammals suffering from diseases caused by Gram-negative bacteria, including bacteremia, endotoxemia, and sepsis. These dual properties of BPI make BPI
10 particularly useful and advantageous for such therapeutic administration.

A proteolytic fragment corresponding to the amino-terminal portion of human BPI possesses the LPS binding and neutralizing activities and antibacterial activity of the naturally-derived 55kD human holoprotein. In contrast to the amino-terminal portion, the carboxyl-terminal region of isolated
15 human BPI displays only slightly detectable antibacterial activity (Ooi *et al.*, 1991, *J. Exp. Med.* 174: 649). One BPI amino-terminal fragment, comprising approximately the first 199 amino acid residues of the human BPI holoprotein and referred to as "rBPI₂₃" (see Gazzano-Santoro *et al.*, 1992, *Infect. Immun.* 60: 4754-4761) has been produced by recombinant means as a 23kD protein.
20 rBPI₂₃ has been introduced into human clinical trials. Proinflammatory responses to endotoxin were significantly ameliorated when rBPI₂₃ was co-administered with LPS.

Other endotoxin binding and neutralizing peptides are known in the art. One example is *Limulus* antilipopolysaccharide factor (LALF) from horseshoe
25 crab amebocytes (Warren *et al.*, 1992, *Infect. Immunol.* 60: 2506-2513). Another example is a cyclic, cationic lipopeptide from *Bacillus polymyxa*, termed Polymyxin B₁. Polymyxin B₁ is composed of six α,γ -diaminobutyric acid residues, one D-phenylalanine, one leucine, one threonine and a 6-methyloctanoyl moiety (Morrison and Jacobs, 1976, *Immunochem.* 13: 813-818)
30 and is also bactericidal. Polymyxin analogues lacking the fatty acid moiety are also known, which analogues retain LPS binding capacity but are without

appreciable bactericidal activity (Danner *et al.*, 1989, *Antimicrob. Agents Chemother.* 33: 1428-1434). Similar properties have also been found with synthetic cyclized polymyxin analogues (Rustici *et al.*, 1993, *Science* 259: 361-365).

5 Known antibacterial peptides include cecropins and magainins. The cecropins are a family of antibacterial peptides found in the hemolymph of lepidopteran insects (Wade *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87: 4761-4765), and the magainins are a family of antibacterial peptides found in *Xenopus* skin and gastric mucosa (Zasloff *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85: 910-913). These peptides are linear and range from about 20 to about 40 amino acids in length. A less active mammalian cecropin has been reported from porcine intestinal mucosa, cecropin P1 (Boman *et al.*, 1993, *Infect. Immun.* 61: 2978-2984). The cecropins are generally reported to be more potent than the magainins in bactericidal activity but appear to have less
10 mammalian cell cytotoxicity. The cecropins and magainins are characterized by a continuous, amphipathic α -helical region which is necessary for bactericidal activity. The most potent of the cecropins identified to date is cecropin A. The sequence of the first ten amino acids of the cecropin A has some homology with the BPI amino acid sequence 90 - 99. However, the
15 other 27 amino acids of cecropin A are clearly necessary for its bactericidal activity and there is little homology with BPI for those 27 amino acids. The magainins have even less homology with the BPI sequence.

 Of interest to the present application are the disclosures in PCT International Application PCT/US91/05758 relating to compositions comprising
25 BPI and an anionic compound, which compositions are said to exhibit (1) no bactericidal activity and (2) endotoxin neutralizing activity. Anionic compounds are preferably a protein such as serum albumin but can also be a polysaccharide such as heparin. In addition, Weiss *et al.* (1975, *J. Clin. Invest.* 55: 33-42) disclose that heparin sulfate and LPS block expression of the
30 permeability-increasing activity of BPI. However, neither reference discloses that BPI actually neutralizes the biologic activities of heparin. Heparin binding

does not necessarily imply heparin neutralization. For example, a family of heparin binding growth factors (HBGF) requires heparin as a cofactor to elicit a biological response. Examples of HBGF's include: fibroblast growth factors (FGF-1, FGF-2) and endothelial cell growth factors (ECGF-1, ECGF-2).
5 Antithrombin III inhibition of clotting cascade proteases is another example of a heparin binding protein that requires heparin for activity and clearly does not neutralize heparin. Heparin binding proteins that do neutralize heparin (*e.g.*, platelet factor IV, protamine, and thrombospondin) are generally inhibitory of the activities induced by heparin binding proteins that use heparin as a
10 cofactor.

BPI (including amino-terminal fragments thereof) has a number of other important biological activities. For example, BPI has been shown to have heparin binding and heparin neutralization activities in copending and co-assigned parent U.S. Patent Application Serial No. 08/030,644 filed March 12,
15 1993 and continuation-in-part U.S. Patent Application Serial No. 08/093,202, filed July 15, 1993, the disclosures of which are incorporated by reference herein. These heparin binding and neutralization activities of BPI are significant due to the importance of current clinical uses of heparin. Heparin is commonly administered in doses of up to 400 U/kg during surgical
20 procedures such as cardiopulmonary bypass, cardiac catheterization and hemodialysis procedures in order to prevent blood coagulation during such procedures. When heparin is administered for anticoagulant effects during surgery, it is an important aspect of post-surgical therapy that the effects of heparin are promptly neutralized so that normal coagulation function can be
25 restored. Currently, protamine is used to neutralize heparin. Protamines are a class of simple, arginine-rich, strongly basic, low molecular weight proteins. Administered alone, protamines (usually in the form of protamine sulfate) have anti-coagulant effects. When administered in the presence of heparin, a stable complex is formed and the anticoagulant activity of both drugs is lost.
30 However, significant hypotensive and anaphylactoid effects of protamine have limited its clinical utility. Thus, due to its heparin binding and neutralization

activities, BPI has potential utility as a substitute for protamine in heparin neutralization in a clinical context without the deleterious side-effects which have limited the usefulness of the protamines. The additional antibacterial and anti-endotoxin effects of BPI would also be useful and advantageous in post-surgical heparin neutralization compared with protamine.

Additionally, BPI is useful in inhibiting angiogenesis due in part to its heparin binding and neutralization activities. In adults, angiogenic growth factors are released as a result of vascular trauma (wound healing), immune stimuli (autoimmune disease), inflammatory mediators (prostaglandins) or from tumor cells. These factors induce proliferation of endothelial cells (which is necessary for angiogenesis) *via* a heparin-dependent receptor binding mechanism (see Yayon *et al.*, 1991, *Cell* 64: 841-848). Angiogenesis is also associated with a number of other pathological conditions, including the growth, proliferation, and metastasis of various tumors; diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation including rheumatoid arthritis, capillary proliferation within atherosclerotic plaques, hemangiomas, endometriosis and Kaposi's sarcoma. Thus, it would be desirable to inhibit angiogenesis in these and other instances, and the heparin binding and neutralization activities of BPI are useful to that end.

Several other heparin neutralizing proteins are also known to inhibit angiogenesis. For example, protamine is known to inhibit tumor-associated angiogenesis and subsequent tumor growth [see Folkman *et al.*, 1992, *Inflammation: Basic Principles and Clinical Correlates*, 2d ed., (Galin *et al.*, eds., Review Press, N.Y.), Ch. 40, pp. 821-839] A second heparin neutralizing protein, platelet factor IV, also inhibits angiogenesis (*i.e.*, is angiostatic). Collagenase inhibitors are also known to inhibit angiogenesis (see Folkman *et al.*, 1992, *ibid.*) Another known angiogenesis inhibitor, thrombospondin, binds to heparin with a repeating serine/tryptophan motif instead of a basic amino acid motif (see Guo *et al.*, 1992, *J. Biol. Chem.* 267: 19349-19355).

Another utility of BPI involves pathological conditions associated with chronic inflammation, which is usually accompanied by angiogenesis. One example of a human disease related to chronic inflammation is arthritis, which involves inflammation of peripheral joints. In rheumatoid arthritis, the inflammation is immune-driven, while in reactive arthritis, inflammation is associated with infection of the synovial tissue with pyogenic bacteria or other infectious agents. Folkman *et al.*, 1992, *supra*, have also noted that many types of arthritis progress from a stage dominated by an inflammatory infiltrate in the joint to a later stage in which a neovascular pannus invades the joint and begins to destroy cartilage. While it is unclear whether angiogenesis in arthritis is a causative component of the disease or an epiphenomenon, there is evidence that angiogenesis is necessary for the maintenance of synovitis in rheumatoid arthritis. One known angiogenesis inhibitor, AGM1470, has been shown to prevent the onset of arthritis and to inhibit established arthritis in collagen-induced arthritis models (Peacock *et al.*, 1992, *J. Exp. Med.* 175: 1135-1138). While nonsteroidal anti-inflammatory drugs, corticosteroids and other therapies have provided treatment improvements for relief of arthritis, there remains a need in the art for more effective therapies for arthritis and other inflammatory diseases.

There continues to exist a need in the art for new products and methods for use as bactericidal agents and endotoxin neutralizing agents, and for heparin neutralization and inhibition of angiogenesis (normal or pathological). One avenue of investigation towards fulfilling this need is the determination of the functional domains of the BPI protein specifying each of these biological activities. Advantageous therapeutic embodiments would therefore comprise BPI functional domain peptides having one or more than one of the activities of BPI.

SUMMARY OF THE INVENTION

This invention provides small, readily-produced peptides having an amino acid sequence that is the amino acid sequence of a BPI functional domain or a subsequence thereof and variants of the sequence or subsequence having at least one of the biological activities of BPI, such as heparin binding, heparin neutralization, LPS binding, LPS neutralization or bactericidal activity. The functional domains of BPI discovered and described herein include: domain I, encompassing the amino acid sequence of BPI from about amino acid 17 to about amino acid 45; domain II, encompassing the amino acid sequence of BPI from about amino acid 65 to about amino acid 99; and domain III, encompassing the amino acid sequence of BPI from about amino acid 142 to about amino acid 169. Thus, the BPI functional domain peptides are based on the amino-terminal portion of human BPI.

The peptides of the invention include linear and cyclized peptides, and peptides that are linear, cyclized and branched-chain combinations of particular BPI functional domain amino acid sequences or subsequences thereof and variants of the sequence or subsequence. Combination peptides include peptides having the sequence or subsequence and variants of the sequence or subsequence of the same or different functional domains of BPI that are covalently linked together. Specifically included are combinations from two to about 10 peptides of any particular sequence or subsequence thereof and variants of that sequence or subsequence. The invention also provides peptides having additional biological activities distinct from the known biological activities of BPI, including but not limited to bactericidal activity having an altered target cell species specificity. Peptides having particular biological properties of BPI that are enhanced or decreased compared with the biological properties of BPI are also provided.

The peptides of the invention include linear and cyclized peptides, and peptides that are linear, cyclized and branched-chain amino acid substitution and additional variants of particular BPI functional domain amino acid sequences or subsequences thereof. For the substitution variants, amino acid

residues at one or more positions in each of the peptides are replaced with a different amino acid residue (including atypical amino acid residues) from that found in the corresponding position of the BPI functional domain from which the specific peptide is derived. For the addition variants, peptides may include up to about a total of 10 additional amino acids, covalently linked to either the amino-terminal or carboxyl-terminal extent, or both, of the BPI functional domain peptides herein described. Such additional amino acids may duplicate amino acids in BPI contiguous to a functional domain or may be unrelated to BPI amino acid sequences and may include atypical amino acids. Linear, cyclized, and branched-chain combination embodiments of the amino acid substitution and addition variant peptides are also provided as peptides of the invention, as are cyclized embodiments of each of the aforementioned BPI functional domain peptides. In addition, peptides of the invention may be provided as fusion proteins with other functional targeting agents, such as immunoglobulin fragments. Addition variants include derivatives and modifications of amino acid side chain chemical groups such as amines, carboxylic acids, alkyl and phenyl groups.

The invention provides pharmaceutical compositions for use in treating mammals for neutralizing endotoxin, killing Gram-negative and Gram-positive bacteria and fungi, neutralizing the anti-coagulant properties of heparin, inhibiting angiogenesis, inhibiting tumor and endothelial cell proliferation, and treating chronic inflammatory disease states. The pharmaceutical compositions comprise unit dosages of the BPI peptides of this invention in solid, semi-solid and liquid dosage forms such as tablet pills, powder, liquid solution or suspensions and injectable and infusible solutions.

This invention provides peptides having an amino acid sequence which is the amino acid sequence of human BPI from about position 17 to about position 45 comprising functional domain I, having the sequence:

Domain I ASQQGTAALQKELKRIKIPDYSDSFKIKH

(SEQ ID NO:1);

and subsequences thereof which have biological activity, including but not limited to one or more of the activities of BPI, for example, bactericidal activity, LPS binding, LPS neutralization, heparin binding or heparin neutralization. Also provided in this aspect of the invention are peptides having substantially the same amino acid sequence of the functional domain I peptides having the amino acid sequence of BPI from about position 17 to about position 45 or subsequences thereof. Additionally, the invention provides peptides which contain two or more of the same or different domain I peptides or subsequence peptides covalently linked together.

This invention provides peptides having an amino acid sequence which is the amino acid sequence of human BPI from about position 65 to about position 99 comprising functional domain II, having the sequence:

Domain II SSQISMVPNVGLKFSISNANIKISGKWKAQKRFLK
(SEQ ID NO:6);

and subsequences thereof which have biological activity, including but not limited to one or more of the activities of BPI, for example, bactericidal activity, LPS binding, LPS neutralization, heparin binding or heparin neutralization. Also provided in this aspect of the invention are peptides having substantially the same amino acid sequence of the functional domain II peptides having the amino acid sequence of BPI from about position 65 to about position 99 or subsequences thereof. Additionally, the invention provides peptides which contain two or more of the same or different domain II peptides or subsequence peptides covalently linked together.

The invention also provides peptides having an amino acid sequence which is the amino acid sequence of human BPI from about position 142 to about position 169 comprising functional domain III, having the sequence:

Domain III VHVHISKSKVGWLIQLFHKKIESALRNK
(SEQ ID NO:12);

and subsequences thereof which have biological activity, including but not limited to one or more of the activities of BPI, for example, bactericidal activity, LPS binding, LPS neutralization, heparin binding or heparin

neutralization. Also provided in this aspect of the invention are peptides having substantially the same amino acid sequence of the functional domain III peptides having the amino acid sequence of BPI from about position 142 to about position 169 or subsequences thereof. Additionally, the invention provides peptides which contain two or more of the same or different domain III peptides or subsequence peptides covalently linked together.

Also provided by this invention are interdomain combination peptides, wherein two or more peptides from different functional domains or subsequences and variants thereof are covalently linked together. Linear, cyclized and branched-chain embodiments of these interdomain combination peptides are provided.

The peptides of this invention have as one aspect of their utility at least one of the known activities of BPI, including LPS binding, LPS neutralization, heparin binding, heparin neutralization and bactericidal activity against Gram-negative bacteria. Additionally and surprisingly, some of the peptides of this invention have utility as bactericidal agents against Gram-positive bacteria. Another surprising and unexpected utility of some of the peptides of this invention is as fungicidal agents. Peptides of this invention provide a new class of antibiotic molecules with the dual properties of neutralizing endotoxin and killing the endotoxin-producing bacteria, useful in the treatment of mammals suffering from diseases or conditions caused by Gram-negative bacteria. Peptides of this invention that retain this dual activity and additionally have an increased antibiotic spectrum represent an additional new class of antimicrobial agents. In addition, peptides of the invention provide a class of antimicrobial agents useful in the treatment of infections by microbial strains that are resistant to traditional antibiotics but are sensitive to the permeability-increasing antimicrobial activity of peptides of the invention.

The invention also provides pharmaceutical compositions of the peptides of the invention comprising the peptides or combinations of the peptides in a pharmaceutically-acceptable carrier or diluent, both *per se* and for use in methods of treating pathological or disease states or for other appropriate

therapeutic uses. Methods of using these pharmaceutical compositions for the treatment of pathological or disease states in a mammal, including humans, are also provided by the invention. Also provided by the invention are uses of BPI functional domain peptide for the manufacture of medicaments for a variety of therapeutic applications.

5

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a and 1b depict HPLC absorbance spectra for cyanogen bromide and proteolytic fragments of rBPI₂₃;

5 Figure 2 is a graph of LAL inhibition assay results for proteolytic fragments of rBPI₂₃;

Figure 3 is a graph of a heparin binding assay results using 15-mer BPI peptides;

Figure 4 is a graph of a *Limulus* Amoebocyte Lysate (LAL) inhibition assay results using 15-mer BPI peptides;

10 Figure 5 is a graph of a radial diffusion bactericidal assay results using 15-mer BPI peptides;

Figure 6 is a graph showing the effect of BPI functional domain peptides in a heparin binding assay;

15 Figures 7a and 7b are graphs showing the effects of BPI functional domain peptides on ATIII/heparin inhibition of thrombin;

Figures 8a and 8b are graphs showing the results of BPI functional domain peptides in an LAL inhibition assay;

Figures 9a, 9b, 9c, and 9d are graphs showing the results of BPI functional domain peptides in radial diffusion bactericidal assays;

20 Figures 9e and 9f are graphs showing the results of BPI functional domain peptides in *E. coli* broth assays;

Figures 10a, 10b, 10c, 10d and 10e are graphs showing the results of BPI functional domain combination peptides in radial diffusion bactericidal assays;

25 Figures 11a, 11b, 11c, 11d, 11e, 11f, 11g, 11h and 11i are graphs showing the results of BPI functional domain peptides in radial diffusion bactericidal assays;

30 Figures 11j and 11k are graphs showing the results of BPI functional domain peptides in bactericidal assays on bacterial cells growing in broth media;

Figure 11l is a graph showing the results of BPI functional domain peptide BPI.30 in bactericidal assays performed in human serum;

Figures 11m and 11n are graphs showing the results of BPI functional domain peptides in radial diffusion bactericidal assays using Gram-positive bacteria;

Figure 11o is a graph showing the results of BPI functional domain peptides in radial diffusion bactericidal assays in comparison with gentamicin and vancomycin using *S. aureus* cells;

Figures 11p and 11q are graphs showing the results of BPI functional domain peptides in cytotoxicity assays using *C. albicans* cells growing in broth media;

Figures 12a, 12b, 12c, 12d, 12e, 12f, and 12g are graphs showing the results of a heparin neutralization assay using BPI functional domain peptides;

Figure 13 is a schematic diagram of the structure of BPI domain II peptide BPI.2 (amino acid sequence 85-99 of the BPI sequence, SEQ ID NO:7);

Figure 14 is a schematic diagram of the structure of BPI domain III peptide BPI.11 (amino acid sequence 148-161 of the BPI sequence, SEQ ID NO:13);

Figures 15a, 15b, 15c, 15d and 15e are graphs showing the results of heparin binding assays using BPI functional domain substitution peptides;

Figure 16 is a graph showing the results of heparin binding experiments using a variety of BPI functional domain peptides;

Figures 17a and 17b are graphs of the results of Lipid A binding competition assays between synthetic BPI functional domain peptides and radiolabeled rBPI₂₃;

Figure 18 is a graph of the results of Lipid A binding competition assays between synthetic BPI.10 peptide and radiolabeled rBPI₂₃ in blood or phosphate buffered saline;

Figure 19 is a graph of the results of Lipid A binding competition assays between synthetic BPI peptides BPI.7, BPI.29 and BPI.30 *versus* radiolabeled rBPI₂₃;

5 Figures 20a and 20b are graphs of the results of Lipid A binding competition assays between BPI functional domain peptides and radiolabeled rLBP₂₅;

Figure 21 is a graph of the results of radiolabeled RaLPS binding experiments using BPI functional domain peptides pre-bound to HUVEC cells;

10 Figures 22a, 22b, 22c, 22d, 22e, 22f, 22g, and 22h are graphs showing the various parameters affecting a cellular TNF cytotoxicity assay measuring the LPS neutralization activity of BPI;

Figures 23a, 23b and 23c are graphs showing the dependence of NO production on the presence of γ -interferon and LBP in LPS-stimulated RAW 264.7 cells and inhibition of such NO production using rBPI₂₃;

15 Figures 24a, 24b, 24c, 24d, 24e, and 24f are graphs showing LPS neutralization by BPI functional domain peptides reflected in their capacity to inhibit NO production by RAW 264.7 cells stimulated by zymosan or LPS;

Figure 24g is a graph showing the IC₅₀ values of synthetic BPI peptides for inhibition of LPS- or zymosan-stimulated NO production by RAW 264.7 cells;

20

Figure 25 is a schematic of rBPI₂₃ showing three functional domains;

Figure 26a is a graph showing the dependence of LPS-mediated inhibition of RAW 264.7 cell proliferation on the presence of rLBP;

25 Figures 26b and 26c are graphs showing patterns of BPI functional domain peptides using the assay of Example 20D;

Figure 27 is a graph showing a comparison of TNF inhibition in whole blood by various BPI functional domain peptides using the assay of Example 20E; and

30 Figure 28 is a graph showing the results of the thrombin clotting time assay described in Example 20G using various BPI functional domain peptides.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides peptides having an amino acid sequence that is the amino acid sequence of at least one functional domain or subsequence thereof and variants of the sequence or subsequence of BPI. For the purposes of this invention, the term "functional domain" is intended to designate a region of the amino acid sequence of BPI that contributes to the total biological activity of the protein. These functional domains of BPI are defined by the activities of proteolytic cleavage fragments, overlapping 15-mer peptides and other synthetic peptides.

Domain I is defined as the amino acid sequence of BPI comprising from about amino acid 17 to about amino acid 45. Peptides based on this domain are moderately active in both the inhibition of LPS-induced LAL activity and in heparin binding assays, and do not exhibit significant bactericidal activity. Domain II is defined as the amino acid sequence of BPI comprising from about amino acid 65 to about amino acid 99. Peptides based on this domain exhibit high LPS and heparin binding capacity and are bactericidal. Domain III is defined as the amino acid sequence of BPI comprising from about amino acid 142 to about amino acid 169. Peptides based on this domain exhibit high LPS and heparin binding activity and are bactericidal.

The functional domains as herein defined include the continuous domains I, II and III, *i.e.*, domains comprised of a continuous portion of the BPI amino acid sequence. However, the invention also includes peptides comprising portions of BPI which are not continuous, *i.e.*, that are separated in the BPI sequence. It is recognized that some non-continuous stretches of amino acid sequence may be folded in the native protein to make such amino acid regions contiguous or in proximity, which structure can be mimicked in the peptides of the invention by covalently linking together peptides from non-continuous regions.

Peptides containing non-continuous regions of BPI amino acid sequence are one example of combination peptides provided by the invention. For the purposes of this invention, combination peptides are intended to include linear,

cyclized or branched-chain peptides comprised of two or more peptides having an amino acid sequence from the same or different functional domains of BPI and subsequences thereof. Specifically encompassed in this definition are combinations containing from two to about 10 functional domain peptides or subsequence thereof, preferably combinations of two or three functional domain peptides (for example, homodimers, homotrimers, heterodimers and heterotrimers). Each of the component peptides comprising such combinations may have an amino acid sequence from any particular BPI functional domain amino acid sequence or subsequence thereof.

For purposes of this invention, the term "a biological activity of BPI" is intended to include, but is not limited to the biological activities of a human bactericidal/permeability-increasing protein (BPI), including, for example, a recombinant BPI holoprotein such as rBPI (SEQ ID NO:69), an amino-terminal fragment of BPI such as rBPI₂₃, and mutated amino-terminal fragments of BPI such as rBPI₂₁Δcys (designated rBPI (1 - 193) ala¹³² in copending and co-assigned U.S. Patent Application Serial No. 08/013,801, filed February 2, 1993, incorporated by reference). As disclosed in copending and co-assigned U.S. Patent Application Serial No. 08/093,202, incorporated by reference, rBPI has been produced having the sequence set out as SEQ ID NO:69 as shown in Gray *et al.* (*supra*) except that valine at position 151 is specified by GTG rather than GTC, and residue 185 is glutamic acid (specified by GAG) rather than lysine (specified by AAG). In addition, rBPI₂₃ (*see also*, Gazzano-Santoro *et al.*, 1992, *Infect. Immun.* 60: 4754-4761) has been produced using an expression vector containing the 31-residue signal sequence and the first 199 amino acids of the sequence of rBPI with the exceptions from the Gray *et al.* (*supra*) sequence as noted above. Such biological activities include LPS binding, LPS neutralization, heparin binding and heparin neutralization, and bactericidal activity. Specifically included is a biological activity of any peptide of this invention that is between 0.1 and 10 times the activity of BPI or of a corresponding peptide encompassing a corresponding functional domain of BPI. Also expressly included in this definition of the "biological activity

of BPI" is a biological activity, for example bactericidal activity, that is qualitatively different than the activity of BPI or the corresponding peptide encompassing the entire corresponding domain of BPI. For example, such qualitative differences include differences in the spectrum of bacteria or other microorganisms against which the peptide is effective, relative to the amino acid sequence of the corresponding functional domain of BPI. This definition thus encompasses peptide activities, such as bactericidal activity against Gram-positive bacteria and fungicidal activity, not previously reported for BPI.

The invention provides peptides each of which has an amino acid sequence that is the amino acid sequence of one of the functional domains of human BPI or a subsequence thereof. Embodiments of such peptides include the following exemplary domain I peptides [single-letter abbreviations for amino acids can be found in G. Zubay, *Biochemistry* (2d. ed.), 1988 (MacMillan Publishing: N.Y.), p.33]:

15	BPI.1	QQGTAALQKELKRIK	(SEQ ID NO:4);
	BPI.4	LQKELKRIKIPDYSDFSFKIKHL	(SEQ ID NO:3);
	BPI.14	GTAALQKELKRIKIPDYSDFSFKIKHLGKGH	(SEQ ID NO:2);
20	and		
	BPI.54	GTAALQKELKRIKIP	(SEQ ID NO:5);

the following exemplary domain II peptides:

25	BPI.2	IKISGKWKAQKRFLK	(SEQ ID NO:7);
	BPI.3	NVGLKFSISNANIKISGKWKAQKRFLK	(SEQ ID NO:11);
	and		
	BPI.8	KWKAQKRFLK	(SEQ ID NO:8);

30 and the following exemplary domain III peptides:

	BPI.5	VHVVHISKSKVGWLIQLFHKKIE	(SEQ ID NO:67);
	BPI.11	KSKVWLIQLFHKK	(SEQ ID NO:13);
	BPI.12	SVHVHISKSKVGWLIQLFHKKIESALRNK	(SEQ ID NO:14);
35	BPI.13	KSKVGWLIQLFHKK	(SEQ ID NO:15);
	and		
	BPI.55	GWLIQLFHKKIESALRNKMNS	(SEQ ID NO:61).

It will be recognized that BPI.14, BPI.12 and BPI.55 are examples of addition variants.

The invention also provides linear and branched-chain combinations of the same or different peptides, wherein each of the peptides of the combination has an amino acid sequence that is the amino acid sequence of one of the functional domains of human BPI or a subsequence thereof. Embodiments of such peptides include the following exemplary combination domain II peptides:

5
10 BPI.9 KRFLKKWKAQKRFLK (SEQ ID NO:51);
BPI.7 KWKAQKRFLKKWKAQKRFLK (SEQ ID NO:54);
BPI.10.1 KRFLKKWKAQKRFLKKWKAQKRFLK (SEQ ID NO:55);
and
15 BPI.10.2 QKRFLKKWKAQKRFLKKWKAQKRFLK (SEQ ID NO:65);

and the following exemplary branched-chain domain II peptide:

MAP.1 (β -alanyl- $N\alpha$, $N\epsilon$ -substituted- $[N\alpha$, $N\epsilon$ (BPI.2)lysyl]lysine);

and the following exemplary combination domain III peptide:

20 BPI.29 KSKVGWLIQLFHKKKSKVGWLIQLFHKK (SEQ ID NO:56);

and the following exemplary branched-chain domain III peptide:

MAP.2 (β -alanyl- $N\alpha$, $N\epsilon$ -substituted- $[N\alpha$, $N\epsilon$ (BPI.13)lysyl]lysine);

25 and the following exemplary domain II-domain III interdomain combination peptides:

BPI.30 KWKAQKRFLKKSKVGWLIQLFHKK (SEQ ID NO:52);
BPI.63 IKISGKWKAQKRFLKKSKVGWLIQLFHKK (SEQ ID NO:53);
30 and
BPI.74 KSKVGWLIQLFHKKKWKAQKRFLK (SEQ ID No.:70).

Amino acid substitution variants are also provided, wherein the amino acid residue at one or more positions in each of the peptides is a residue different from the amino acid found in the corresponding position of the BPI functional domain from which that specific peptide is derived. For example, in one embodiment of this aspect of the invention, one position in the peptide is substituted with an alanine residue for the amino acid found at the

corresponding position in the BPI amino acid sequence. In other embodiments, one position in the peptide is substituted with *e.g.*, a phenylalanine, leucine, lysine or tryptophan residue for the amino acid found at the corresponding position in the BPI amino acid sequence. Embodiments of these peptides include the following exemplary substitution domain II peptides:

5	BPI.15	AKISGKWKAQKRFLK	(SEQ ID NO:16);
	BPI.16	IAISGKWKAQKRFLK	(SEQ ID NO:17);
	BPI.17	IKASGKWKAQKRFLK	(SEQ ID NO:18);
10	BPI.18	IKIAGKWKAQKRFLK	(SEQ ID NO:19);
	BPI.19	IKISAKWKAQKRFLK	(SEQ ID NO:20);
	BPI.20	IKISGAWKAQKRFLK	(SEQ ID NO:21);
	BPI.21	IKISGKAKAQKRFLK	(SEQ ID NO:22);
	BPI.22	IKISGKWAAQKRFLK	(SEQ ID NO:23);
15	BPI.23	IKISGKWKAQKRFLK	(SEQ ID NO:24);
	BPI.24	IKISGKWKAQARFLK	(SEQ ID NO:25);
	BPI.25	IKISGKWKAQKAFLK	(SEQ ID NO:26);
	BPI.26	IKISGKWKAQKRALK	(SEQ ID NO:27);
	BPI.27	IKISGKWKAQKRFAK	(SEQ ID NO:28);
20	BPI.28	IKISGKWKAQKRFLA	(SEQ ID NO:29);
	BPI.61	IKISGKFKAQKRFLK	(SEQ ID NO:48);
	BPI.73	IKISGKWKAQFRFLK	(SEQ ID NO:62);
	BPI.77	IKISGKWKAQWRFLK	(SEQ ID NO:72);
	BPI.79	IKISGKWKAKKRFLK	(SEQ ID NO:73);
25	and		
	BPI.81	IKISGKWKAQKRFLK	(SEQ ID NO:75);

and the following exemplary substitution domain III peptides:

30	BPI.31	ASKVGWLIQLFHKK	(SEQ ID NO:33);
	BPI.32	KAKVGWLIQLFHKK	(SEQ ID NO:34);
	BPI.33	KSAVGWLIQLFHKK	(SEQ ID NO:35);
	BPI.34	KSKAGWLIQLFHKK	(SEQ ID NO:36);
	BPI.35	KSKVAWLIQLFHKK	(SEQ ID NO:37);
35	BPI.36	KSKVGALIQLFHKK	(SEQ ID NO:38);
	BPI.37	KSKVGWAIQLFHKK	(SEQ ID NO:39);
	BPI.38	KSKVGWLAQLFHKK	(SEQ ID NO:40);
	BPI.39	KSKVGWLIQLFHKK	(SEQ ID NO:41);
	BPI.40	KSKVGWLIQAFHKK	(SEQ ID NO:42);
40	BPI.41	KSKVGWLIQLAHKK	(SEQ ID NO:43);
	BPI.42	KSKVGWLIQLFAKK	(SEQ ID NO:44);
	BPI.43	KSKVGWLIQLFHAK	(SEQ ID NO:45);
	BPI.44	KSKVGWLIQLFHKA	(SEQ ID NO:46);
	BPI.82	KSKVGWLIQLWHKK	(SEQ ID NO:76);
45	BPI.85	KSKVLWLIQLFHKK	(SEQ ID NO:79);

BPI.86 KSKVGWLILLFHKK (SEQ ID NO:80);
 BPI.87 KSKVGWLIQLFLKK (SEQ ID NO:81);
 BPI.91 KSKVGWLIFLFHKK (SEQ ID NO:86);
 BPI.92 KSKVGWLIKLFHKK (SEQ ID NO:87);
 5 BPI.94 KSKVGWLIQLFFKK (SEQ ID NO:89);
 BPI.95 KSKVFWLIQLFHKK (SEQ ID NO:90);
 BPI.96 KSKVGWLIQLFHKF (SEQ ID NO:91);
 and
 BPI.97 KSKVKWLIQLFHKK (SEQ ID NO:92).

10 A particular utility of such single amino acid-substituted BPI functional domain
 peptides provided by the invention is to identify critical residues in the peptide
 sequence, whereby substitution of the residue at a particular position in the
 amino acid sequence has a detectable effect on at least one of the biological
 activities of the peptide. Expressly encompassed within the scope of this
 15 invention are embodiments of the peptides of the invention having substitutions
 at such critical residues so identified using any amino acid, whether naturally-
 occurring or atypical, wherein the resulting substituted peptide has biological
 activity as defined herein.

Substituted peptides are also provided that are multiple substitutions,
 20 *i.e.*, where two or more different amino acid residues in the functional domain
 amino acid sequence are each substituted with another amino acid. For
 example, in embodiments of such doubly-substituted peptides, both positions in
 the peptide are substituted *e.g.*, with alanine, phenylalanine or lysine residues
 for the amino acid found at the corresponding positions in the BPI amino acid
 25 sequence. Examples of embodiments of these peptides include the multiply
 substituted domain II peptides:

BPI.45 IKISGKWKAAARFLK (SEQ ID NO:31);
 BPI.56 IKISGKWKAKQRFLK (SEQ ID NO:47);
 30 BPI.59 IKISGAWAAQKRFLK (SEQ ID NO:30);
 BPI.60 IAISGKWKAQKRFLA (SEQ ID NO:32);
 and
 BPI.88 IKISGKWKAFFRFLK (SEQ ID NO:82);

35 and the exemplary multiply substituted domain III peptide:

BPI.100 KSKVKWLIKLFHKK (SEQ ID NO:94);

and the following exemplary multiply substituted domain II substitution combination peptide:

BPI.101 KSKVKWLIKLFKFKSKVKWLIKLFKFK
(SEQ ID NO:95);

5 and the following exemplary multiply substituted domain II-domain III interdomain substitution combination peptide:

BPI.102 KWKAQFRFLKKSKVGWLIILFHKK
(SEQ ID NO:96).

10 Another aspect of such amino acid substitution variants are those where the substituted amino acid residue is an atypical amino acid. Specifically encompassed in this aspect of the peptides of the invention are peptides containing D-amino acids, modified or non-naturally-occurring amino acids, and altered amino acids to provide peptides with increased stability, potency or
15 bioavailability. Embodiments of these peptides include the following exemplary domain II peptides with atypical amino acids:

	BPI.66	IKISGKW _D KAQKRFLK	(SEQ ID NO:49);
	BPI.67	IKISGKA _{β-(1-naphthyl)} KAQKRFLK	(SEQ ID NO:50);
20	BPI.70	IKISGKA _{β-(3-pyridyl)} KAQKRFLK	(SEQ ID NO:63);
	BPI.71	A _D A _D IKISGKWKAQKRFLK	(SEQ ID NO:66);
	BPI.72	IKISGKWKAQKRA _{β-(3-pyridyl)} LK	(SEQ ID NO:64);
	BPI.76	IKISGKWKAQF _D RFLK	(SEQ ID NO:71);
	BPI.80	IKISGKWKAQA _{β-(1-naphthyl)} RFLK	(SEQ ID NO:74);
25	BPI.84	IKISGKA _{β-(1-naphthyl)} KAQFRFLK	(SEQ ID NO:78);
	BPI.89	IKISGKA _{β-(1-naphthyl)} KAFKRFLK	(SEQ ID NO:84);
	and		
	BPI.90	IKISGKA _{β-(1-naphthyl)} KAFFRFLK	(SEQ ID NO:85);

30 the exemplary domain III peptide with atypical amino acids:

BPI.83 KSKVGA _{β -(1-naphthyl)}LIQLFHKK (SEQ ID NO:77);

35 and the exemplary domain II-domain III interdomain combination peptides with atypical amino acids:

	BPI.93	IKISGKA _{β-(1-naphthyl)} KAQFRFLKKSKVGWLIQLFHKK	(SEQ ID NO:88);
	and		
40	BPI.98	IKISGKA _{β-(1-naphthyl)} KAQFRFLKKSKVGWLIQLFHKK	(SEQ ID NO:83).

Linear and branched-chain combination embodiments of the amino acid substitution variant peptides, which create multiple substitutions in multiple domains, are also an aspect of this invention. Embodiments of these peptides include the following exemplary combination/substitution domain II peptides:

5
BPI.46 KWKAAARFLKKWKAQRFLK (SEQ ID NO:57);
BPI.47 KWKAQKRFLKKWKAARFLK (SEQ ID NO:58);
BPI.48 KWKAAARFLKKWAAAKRFLK (SEQ ID NO:59);
10 BPI.69 KWKAAARFLKKWKAARFLKKWKAARFLK (SEQ ID NO:60);
and
BPI.99 KWKAQWRFLKKWKAQWRFLKKWKAQWRFLK (SEQ ID NO:93).

15 Dimerized and cyclized embodiments of each of the aforementioned BPI functional domain peptides are also provided by this invention. Embodiments of these peptides include the following exemplary cysteine-modified domain II peptides:

	BPI.58	CIKISGKWKAQKRFLK	(SEQ ID NO: 9);
20	BPI.65(red)	CIKISGKWKAQKRFLKC	(SEQ ID NO:68);
	and		
	BPI.65(ox.)	<div style="display: flex; align-items: center;"> <div style="border-top: 1px solid black; width: 100px; margin-right: 5px;"></div> <div style="text-align: center; margin-right: 5px;">S - S</div> <div style="border-top: 1px solid black; width: 100px; margin-left: 5px;"></div> </div> CIKISGKWKAQKRFLKC	(SEQ ID NO:10).

BPI functional domain peptides described herein are useful as potent anti-bacterial agents for Gram-negative bacteria and for neutralizing the adverse effects of LPS associated with the cell membranes of Gram-negative bacteria. The peptides of the invention have, in varying amounts, additional activities of BPI, including activities not directly associated with the Gram-negative bacterial infection, such as heparin binding and neutralization. Peptides provided by this invention also may have biological activities distinct from the known biological activities of BPI. For example, some embodiments of the peptides of the invention surprisingly have been found to have a biological target range for bactericidal activity that is broader than BPI and exhibits bactericidal activity against Gram-positive as well as Gram-negative bacteria. Some embodiments of the invention have surprisingly been found to have fungicidal activity. Thus, the invention advantageously provides peptides having amino acid sequences of the biologically functional domains of BPI having distinct

antimicrobial activities. Peptides of this invention that possess the dual anti-bacterial and anti-endotoxic properties of BPI, including those with an increased antibiotic spectrum, represent a new class of antibiotic molecules.

5 BPI functional domain peptides of the invention will have biological therapeutic utilities heretofor recognized for BPI protein products. For example, co-owned, copending U.S. Patent Application Serial No. 08/188,221 filed January 24, 1994, addresses use of BPI protein products in the treatment of humans exposed to Gram-negative bacterial endotoxin in circulation. Co-owned, copending U.S. Patent Application Serial No. 08/031,145 filed March 10 12, 1993 addresses administration of BPI protein products for treatment of mycobacterial diseases. Co-owned, copending U.S. Patent Application Serial No. 08/132,510, filed October 5, 1993, addresses use of BPI protein products in the treatment of conditions involving depressed reticuloendothelial system function. Co-owned, copending U.S. Patent Application Serial No. 15 08/125,651, filed September 22, 1993, addresses synergistic combinations of BPI protein products and antibiotics. Co-owned, copending U.S. Patent Application Serial No. 08/093,201 filed July 14, 1993, addresses methods of potentiating BPI protein product bactericidal activity by administration of LBP protein products. Co-owned, copending U.S. Patent Application Serial No. 20 08/031,144 filed March 12, 1993, addresses administration of BPI protein products for treatment of Helicobacterial infections. The disclosures of the above applications are specifically incorporated by reference herein for the purpose of exemplifying therapeutic uses for BPI functional domain peptides of the invention. The BPI functional domain peptides of the invention also have 25 therapeutic utility for the treatment of pathological conditions and disease states as disclosed in the above identified U.S. Patent Application Serial Nos. 08/030,644, 08/093,202 and 08/183,222 parent applications.

BPI functional domain peptides of the invention are thus useful in methods for: neutralizing the anti-coagulant effect of heparin; inhibiting 30 angiogenesis (especially angiogenesis associated with ocular retinopathy); inhibiting endothelial cell proliferation (especially endometriosis and

proliferation associated with implantation of fertilized ova); inhibiting malignant tumor cell proliferation (especially Kaposi's sarcoma proliferation); treating chronic inflammatory disease states (such as arthritis and especially reactive and rheumatoid arthritis); treating Gram-negative bacterial infection and the sequelae thereof; treating the adverse effects (such as increased cytokine production) of Gram-negative endotoxin in blood circulation; killing Gram-negative bacteria; treating adverse physiological effects associated with depressed reticuloendothelial system function (especially involving depressed function of Kupffer cells of the liver such as results from physical, chemical and biological insult to the liver); treating, in synergistic combination with antibiotics (such as gentamicin, polymyxin B and cefamandole nafate) Gram-negative bacterial infection and the sequelae thereof; killing Gram-negative bacteria in synergistic combination with antibiotics; treating, in combination with LBP protein products, Gram-negative bacterial infection and the sequelae thereof; killing Gram-negative bacteria in combination with LBP protein products; treating, alone or in combination with antibiotics and/or bismuth, *Mycobacteria* infection (especially infection by *M. tuberculosis*, *M. leprae* and *M. avium*); treating adverse physiological effects (such as increased cytokine production) of lipoarabinomannan in blood circulation; decontaminating fluids (such as blood, plasma, serum and bone marrow) containing lipoarabinomannan; and, treating disease states (such as gastritis and peptic, gastric and duodenal ulcers) associated with infection by bacteria of the genus *Helicobacter*. The present invention also provides pharmaceutical compositions for oral, parenteral, topical and aerosol administration comprising BPI functional domain peptides in amounts effective for the uses noted above and especially compositions additionally comprising pharmaceutically acceptable diluents, adjuvants or carriers.

With respect to uses of BPI functional domain peptides in combination with LBP protein products, as used herein, "LBP protein product" includes naturally and recombinantly product lipopolysaccharide binding protein; natural, synthetic, and recombinant biologically active polypeptide fragments and

derivatives of lipopolysaccharide binding protein; and biologically active polypeptide analogs, including hybrid fusion proteins, of either LBP or biologically active fragments thereof. LBP protein products useful according to the methods of the present invention include LBP holoprotein which can be produced by expression of recombinant genes in transformed eucaryotic host cells such as described in co-owned and copending U.S. Patent Application Serial No. 08/079,510 filed June 17, 1993 and designated rLBP. Also described in that application are preferred LBP protein derivatives which lack CD14-mediated inflammatory properties and particularly the ability to mediate LPS activity through the CD14 receptor. Such LBP protein products are preferred for use according to the present invention because excessive CD14-mediated immunostimulation is generally considered undesirable, and is particularly so in subjects suffering from infection.

Preferred LBP protein derivatives are characterized as amino-terminal fragments having a molecular weight of about 25kD. Most preferred are LBP amino-terminal fragments characterized by the amino acid sequence of the first 197 amino acids of the amino-terminus of LBP, as set out in SEQ ID NOS:97 and 98, designated rLBP₂₅, the production of which is described in previously-noted co-owned and copending U.S. Patent Application Serial No. 08/079,510. It is contemplated that LBP protein derivatives considerably smaller than 25 kD and comprising substantially fewer than the first 197 amino acids of the amino-terminus of the holo-LBP molecule are suitable for use according to the invention provided they retain the ability to bind to LPS. Moreover, it is contemplated that LBP protein derivatives comprising greater than the first 197 amino acid residues of the holo-LBP molecule including amino acids on the carboxy-terminal side of first 197 amino acids of the rLBP as disclosed in SEQ ID NOS: 97 and 98 will likewise prove useful according to the methods of the invention provided they lack an element that promotes CD14-mediated immunostimulatory activity. It is further contemplated that those of skill in the art are capable of making additions, deletions and substitutions of the amino acid residues of SEQ ID NOS: 97 and 98 without loss of the desired

biological activities of the molecules. Still further, LBP protein products may be obtained by deletion, substitution, addition or mutation, including mutation by site-directed mutagenesis of the DNA sequence encoding the LBP holoprotein, wherein the LBP protein product maintains LPS-binding activity and lacks CD14-mediated immunostimulatory activity. Specifically contemplated are LBP hybrid molecules and dimeric forms which may result in improved affinity of LBP for bacteria and/or increased stability *in vivo*. These include LBP/BPI hybrid proteins and LBP-Ig fusion proteins. Such hybrid proteins further include those using human gamma 1 or gamma 3 hinge regions to permit dimer formation. Other forms of dimer contemplated to have enhanced serum stability and binding affinity include fusions with Fc lacking the CH₂ domain, or hybrids using leucine or helix bundles.

BPI functional domain peptides of the invention may be generated and/or isolated by any means known in the art, including by means of recombinant production. Co-owned U.S. Patent No. 5,028,530, issued July 2, 1991, co-owned U.S. Patent No. 5,206,154, issued April 27, 1993, and co-owned, copending U.S. Patent Application Serial No. 08/010,676, filed January 28, 1993, all of which are hereby incorporated by reference, disclose novel methods for the recombinant production of polypeptides, including antimicrobial peptides. Additional procedures for recombinant production of antimicrobial peptides in bacteria have been described by Piers *et al.*, 1993, *Gene* 134: 7-13. Co-owned, copending U.S. Patent Application Serial No. 07/885,501, filed May 19, 1992, and a continuation-in-part thereof, U.S. Patent Application Serial No. 08/072,063, filed May 19, 1993 which are both hereby incorporated by reference, disclose novel methods for the purification of recombinant BPI expressed in and secreted from genetically transformed mammalian host cells in culture and discloses how one may produce large quantities of recombinant BPI suitable for incorporation into stable, homogeneous pharmaceutical preparations.

BPI functional domain peptides may also be advantageously produced using any such methods. Those of ordinary skill in the art are able to isolate

or chemically synthesize a nucleic acid encoding each of the peptides of the invention. Such nucleic acids are advantageously utilized as components of recombinant expression constructs, wherein the nucleic acids are operably linked with transcriptional and/or translational control elements, whereby such recombinant expression constructs are capable of expressing the peptides of the invention in cultures of prokaryotic, or preferably eukaryotic cells, most preferably mammalian cells, transformed with such recombinant expression constructs.

Peptides of the invention may be advantageously synthesized by any of the chemical synthesis techniques known in the art, particularly solid-phase synthesis techniques, for example, using commercially-available automated peptide synthesizers. Such peptides may also be provided in the form of combination peptides, wherein the peptides comprising the combination are linked in a linear fashion one to another and wherein a BPI sequence is present repeatedly in the peptide, with or without separation by "spacer" amino acids allowing for selected conformational presentation. Also provided are branched-chain combinations, wherein the component peptides are covalently linked *via* functionalities in amino acid sidechains of the amino acids comprising the peptides.

Functional domain peptides of this invention can be provided as recombinant hybrid fusion proteins comprising BPI functional domain peptides and at least a portion of at least one other polypeptide. Such proteins are described, for example, by Theofan *et al.* in co-owned, copending U.S. Patent Application Serial No. 07/885,911, filed May 19, 1992, and a continuation-in-part application thereof, U.S. Patent Application Serial No. 08/064,693, filed May 19, 1993, which are incorporated herein by reference in their entirety.

Generally, those skilled in the art will recognize that peptides as described herein may be modified by a variety of chemical techniques to produce compounds having essentially the same activity as the unmodified peptide, and optionally having other desirable properties. For example, carboxylic acid groups of the peptide, whether carboxyl-terminal or sidechain,

may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C_1 - C_{16} ester, or converted to an amide of formula NR_1R_2 wherein R_1 and R_2 are each independently H or C_1 - C_{16} alkyl, or combined to form a heterocyclic ring, such as 5- or 6-membered. Amino groups of the peptide, whether amino-terminal or sidechain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C_1 - C_{16} alkyl or dialkyl amino or further converted to an amide. Hydroxyl groups of the peptide sidechain may be converted to C_1 - C_{16} alkoxy or to a C_1 - C_{16} ester using well-recognized techniques. Phenyl and phenolic rings of the peptide sidechain may be substituted with one or more halogen atoms, such as fluorine, chlorine, bromine or iodine, or with C_1 - C_{16} alkyl, C_1 - C_{16} alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide sidechains can be extended to homologous C_2 - C_4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides of this invention to select and provide conformational constraints to the structure that result in enhanced binding and/or stability. For example, a carboxyl-terminal or amino-terminal cysteine residue can be added to the peptide, so that when oxidized the peptide will contain a disulfide bond, thereby generating a cyclic peptide. Other peptide cyclizing methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

Peptidomimetic and organomimetic embodiments are also hereby explicitly declared to be within the scope of the present invention, whereby the three-dimensional arrangement of the chemical constituents of such peptido- and organomimetics mimic the three-dimensional arrangement of the peptide backbone and component amino acid sidechains in the peptide, resulting in such peptido- and organomimetics of the peptides of this invention having substantial biological activity. It is implied that a pharmacophore exists for each of the described activities of BPI. A pharmacophore is an idealized, three-

dimensional definition of the structural requirements for biological activity. Peptido- and organomimetics can be designed to fit each pharmacophore with current computer modelling software (computer aided drug design). The degree of overlap between the specific activities of pharmacophores remains to be determined.

5 The administration of BPI functional domain peptides is preferably accomplished with a pharmaceutical composition comprising a BPI functional domain peptide and a pharmaceutically acceptable diluent, adjuvant, or carrier. The BPI functional domain peptide composition may be administered without
10 or in conjunction with known antibiotics, surfactants, or other chemotherapeutic agents. Examples of such combinations are described in co-owned, copending, U.S. Patent Application Serial No. 08/012,360, filed February 2, 1993, and continuation-in-part U.S. Patent Application Serial No. 08/190,869, filed February 2, 1994, the disclosures of which are incorporated herein by
15 reference.

Effective doses of BPI functional domain peptides for bactericidal activity, partial or complete neutralization of the anti-coagulant activity of heparin, partial or complete neutralization of LPS and other effects described herein may be readily determined by those of skill in the art according to
20 conventional parameters, each associated with the corresponding biological activity, including, for example, the size of the subject, the extent and nature of the bacterial infection, the extent and nature of the endotoxic shock, and the quantity of heparin administered to the subject and the time since administration of the heparin. Similar determinations will be made by those of skill in this
25 art for using the peptide embodiments of this invention for therapeutic uses envisioned and described herein.

Embodiments of the invention comprising medicaments can be prepared for oral administration, for injection, or other parenteral methods and preferably include conventional pharmaceutically acceptable carriers, adjuvants
30 and counterions as would be known to those of skill in the art. The medicaments are preferably in the form of a unit dose in solid, semi-solid and

liquid dosage forms such as tablets, pills, powders, liquid solutions or suspensions, and injectable and infusible solutions. Effective dosage ranges from about 100 $\mu\text{g/kg}$ to about ¹⁰⁰~~10~~ mg/kg of body weight are contemplated.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. Example 1 describes the preparation of proteolytic fragments of BPI; Example 2 describes the results of bactericidal assays of the proteolytic fragments of Example 1; Example 3 describes the results of heparin binding assays using the proteolytic fragments of Example 1; Example 4 describes the results of experiments using *Limulus* amebocyte lysates to assay the LPS binding activity of the proteolytic fragments of Example 1; Example 5 describes the preparation of 15-mer peptides of BPI; Example 6 describes the results of heparin binding assays using the 15-mer peptides of Example 5; Example 7 describes the results of *Limulus* amebocyte lysates assays using the 15-mer peptides of Example 5; Example 8 describes the results of bactericidal assays of the 15-mer peptides of Example 5; Example 9 describes the preparation of BPI individual functional domain peptides; Example 10 describes the results of heparin binding assays using the BPI individual functional domain peptides of Example 9; Example 11 describes the results of heparin neutralization assays using the BPI individual functional domain peptides of Example 9; Example 12 describes the results of *Limulus* amebocyte lysates assays of LPS neutralization activity using the BPI individual functional domain peptides of Example 9; Example 13 describes the results of bactericidal assays of the BPI individual functional domain peptides of Example 9; Example 14 describes the preparation of BPI combination functional domain peptides; Example 15 describes the results of bactericidal activity assays of the BPI combination functional domain peptides of Example 14; Example 16 describes the results of additional bactericidal activity assays of the BPI combination functional domain peptides of Example 14; Example 17 describes the results of *in vivo* and *in vitro* heparin neutralization assays using the BPI combination functional domain peptides of Example 14; Example 18 describes the preparation and functional activity analysis of bactericidal activity, heparin

binding activity and LPS neutralization activity assays of BPI substitution variant functional domain peptides; Example 19 provides a summary of the results of bactericidal and heparin binding assays using representative BPI functional domain peptides; Example 20 describes analysis of BPI functional domain peptides in a variety of binding and neutralization assays; Example 21 addresses a heparin neutralization assay; Example 22 describes administration of BPI functional domain peptides in model systems of collagen and bacteria-induced arthritis animal model systems exemplifying treatment of chronic inflammatory disease states; Example 23 illustrates testing of BPI functional domain peptides for angiostatic effects in a mouse malignant melanoma metastasis model system; Example 24 addresses effects of BPI functional domain peptides on endothelial cell proliferation; Example 25 describes analysis of BPI functional domain peptides in animal model systems; and Example 26 describes a protocol for testing the anti-endotoxin effects of BPI functional domain peptides of the invention *in vivo* in humans.

EXAMPLE 1**Preparation of BPI Proteolytic Fragments**

Chemical cleavage and enzymatic digestion processes were applied to rBPI₂₃ to produce variously-sized proteolytic fragments of the recombinant BPI protein.

rBPI₂₃ protein was reduced and alkylated prior to proteolysis by cyanogen bromide (CNBr) or endoproteinase Asp-N. The protein was desalted by overnight precipitation upon the addition of cold (4°C) acetone (1:1 v/v) and the precipitated protein recovered by pelleting under centrifugation (5000 x g) for 10 minutes. The rBPI₂₃ protein pellet was washed twice with cold acetone and dried under a stream of nitrogen. An rBPI₂₃ solution was then reconstituted to a final concentration of 1 mg protein/mL in 8M urea/0.1M Tris-HCl (pH 8.1) and reduced by addition of 3.4 mM dithiothreitol (Calbiochem, San Diego, CA) for 90 minutes at 37°C. Alkylation was performed by the addition of iodoacetamide (Sigma Chemical Co., St. Louis, MO) to a final concentration of 5.3 millimolar and incubation for 30 minutes in the dark at room temperature. The reduced and alkylated protein was acetone-precipitated, centrifuged and washed as described above and the pellet was redissolved as described below for either CNBr or Asp-N digestion.

For CNBr-catalyzed protein fragmentation, the washed pellet was first dissolved in 70% trifluoroacetic acid (TFA) (Protein Sequencing Grade, Sigma Chemical Co., St. Louis, MO) to a final protein concentration of 5 mg/mL. Cyanogen bromide (Baker Analyzed Reagent, VWR Scientific, San Francisco, CA) dissolved in 70% TFA was added to give a final ratio of 2:1 CNBr to protein (w/w). This ratio resulted in an approximately 75-fold molar excess of CNBr relative to the number of methionine residues in the rBPI₂₃ protein. The reaction was purged with nitrogen and allowed to proceed for 24 hours in the dark at room temperature. The reaction was terminated by adding 9 volumes of distilled water, and followed by freezing (-70°C) and lyophilization.

For endoproteinase digestion, the reduced and alkylated rBPI₂₃ was solubilized at a concentration of 5.0 mg/mL in 8M urea/0.1M Tris-HCl (pH

8.1). An equal volume of 0.1M Tris-HCl (pH 8.1) was then added so that the final conditions were 2.5 mg/mL protein in 5M urea/0.1M Tris-HCl (pH 8.1). Endoproteinase Asp-N from *Pseudomonas fragi* (Boehringer-Mannheim, Indianapolis, IN) was added at a 1:1000 (w/w, enzyme:substrate) ratio, and digestion was allowed to proceed for 6 hours at 37°C. The reaction was terminated by addition of TFA to a final concentration of 0.1% and the samples were then fractionated by reverse phase HPLC.

The CNBr and Asp-N fragment mixtures were purified on a Zorbax Protein Plus C₃ column (4.6 x 250 mm, 300 Å pore size, MACMOD Analytical Inc, Chadsford, PA). A gradient ranging from 5% acetonitrile in 0.1% TFA to 80% acetonitrile in 0.1 % TFA was run over this column over a 2 hour elution period at a flow rate of 1.0 mL/min. Fragment elution was monitored at 220 nm using a Beckman System Gold HPLC (Beckman Scientific Instruments, San Ramon, CA). The column heating compartment was maintained at 35°C and the fractions were collected manually, frozen at -70°C and dried in a Speed Vac concentrator. Fragments were then solubilized in a solution of 20 mM sodium acetate (pH 4.0)/0.5 M NaCl prior to use.

Electrospray ionization mass spectrometry (ESI-MS) was performed on a VG Bio-Q mass spectrometer by Dr. Francis Bitsch and Mr. John Kim in the laboratory of Dr. Cedric Shackleton, Children's Hospital-Oakland Research Institute. Molecular masses were obtained by mathematical transformation of the data.

Although the DNA sequence for rBPI₂₃ encodes amino acid residues 1-199 of the mature protein, a significant portion of the protein that is produced is truncated at Leu-193 and Val-195, as determined by ESI-MS. The existence of these carboxyl-terminal truncations were verified by isolating the carboxyl-terminal tryptic peptides, which were sequenced and analyzed by ESI-MS.

There are six methionine residues in the rBPI₂₃ protein, at positions 56, 70, 100, 111, 170, and 196, and chemical cleavage by cyanogen bromide produced six major peptide fragments as predicted. The results of the CNBr cleavage experiments are summarized in Table I. The fragments were isolated

by reverse phase (C_3) HPLC (Figure 1a) and their amino-terminal sequences were determined by Edman degradation. The two largest fragments (C1 and C5) were not resolved by the C_3 HPLC column and further attempts to resolve them by ion exchange chromatography were unsuccessful, presumably because they are similar in length and isoelectric point. The identities of the C1, C5 fragments within the mixture were determined by ESI-MS. The predicted mass of C1 is 6269 (Table I), taking into account the loss of 30 a.m.u. resulting from the conversion of the carboxyl-terminal methionine to homoserine during the CNBr cleavage reaction. The observed mass of 6251.51 ± 0.34 is consistent with the loss of a water molecule (18 a.m.u.) in a homoserine lactone intermediate, which may be favored over the formation of the homoserine because of the hydrophobicity of the C1 fragment C-terminal amino acids. The predicted mass of the C5 fragment is 6487 and the observed mass is 6385.84 ± 0.39 (Table I). For the C5 fragment, the C-terminal amino acids are hydrophilic, so the hydrolysis of the homoserine lactone intermediate is probably favored. From both the amino-terminal sequencing and the mass spectrum data, the C5 component represents approximately 10-25% of the material in the C1/C5 mixture.

Proteolytic cleavage with endoproteinase Asp-N was performed to provide additional fragments for the regions contained within the CNBr C1/C5 mixture. There are six aspartic acid residues within the rBPI₂₃ sequence at positions 15, 36, 39, 57, 105, and 116. The six major Asp-N fragments isolated by C_3 HPLC (Figure 1b) were sequenced and masses were determined by ESI-MS (Table I). A short duration digest at a 1:1000 (w/w, enzyme:substrate) ratio was used to eliminate potential non-specific cleavages, particularly at glutamic acid residues. It is evident that this digestion did not continue until completion, as one fragment (1-38) was isolated where Asp residues (amino acids 15 and 35) were not cleaved. The mass spectra of the Asp-N fragments were consistent with the predicted masses for each individual fragment. Unlike the CNBr cleavage, where the carboxyl-terminal fragment

was poorly resolved, the Asp-N fragment from amino acid 116 to the carboxyl-terminus was well resolved from all of the other Asp-N fragments.

TABLE I
Summary of rBPI₂₃ Cleavage Fragment Analysis

<u>CNBr Cleavage Fragments</u>			MASS	
<u>PEAK</u>	<u>SEQUENCE</u>	<u>I.D.</u>	<u>measured</u>	<u>predicted</u>
I	101-110	C4(101-111)	N.D.	1169
II	57-67	C2(57-70)	N.D.	1651
III	71-99	C3(71-100)	N.D.	3404
IV	171-194	C6(171-196)	N.D.	2929
V	1-25, 112-124	C1(1-56), C5(112-170)	6251 6486	6269 6487
<u>Asp-N Proteolytic Fragments</u>			MASS	
<u>PEAK</u>	<u>SEQUENCE</u>	<u>I.D.</u>	<u>measured</u>	<u>predicted</u>
A	1-14	A1(1-14)	1465.5	1464
I	39-56	A3(39-56)	2145.2	2145
II	15-38	A2(15-38)	2723.6	2724
III	57-76	A4(57-104)	5442.5	5442
IV	1-38	A1 A2(1-38)	4171.4	4172
VI	116-134	A6a(116-193)	8800.3	8800
VII	116-128	A6b(116-195)	8997.1	8996

EXAMPLE 2

Bactericidal Effects of BPI Proteolytic Fragments

BPI proteolytic fragments produced according to Example 1 were screened for bactericidal effects using rough mutant *E. coli* J5 bacteria in a radial diffusion assay. Specifically, an overnight culture of *E. coli* J5 was diluted 1:50 into fresh tryptic soy broth and incubated for 3 hours at 37°C to attain log phase growth of the culture. Bacteria were then pelleted at 3,000 rpm for 5 minutes in a Sorvall RT6000B centrifuge (Sorvall Instruments, Newton, CT). 5 mL of 10 mM sodium phosphate buffer (pH 7.4) was added and the preparation was re-pelleted. The supernatant was decanted and 5 mL of fresh buffer was added, the bacteria were resuspended and their

concentration was determined by measurement of absorbance at 590 nm (an Absorbance value of 1.00 at this wavelength equals a concentration of 1.25×10^9 CFU/mL in suspension). The bacteria were diluted to 4×10^6 CFU/mL in 10 mL of molten underlayer agarose (at approximately 45°C) and inverted repeatedly to mix in 15 mL polypropylene tubes conventionally used for this purpose.

The entire contents of such tubes were then poured into a level square petri dish and distributed evenly by rocking the dish side-to-side. The agarose hardened in less than 30 seconds and had a uniform thickness of about 1 mm. A series of wells were then punched into the hardened agarose using a sterile 3 mm punch attached to a vacuum apparatus. The punch was sterilized with 100% alcohol and allowed to air dry prior to use to avoid contaminating the bacterial culture.

5 or 10 μ L of each of the BPI fragments were carefully pipetted into each well. As a negative control, dilution buffer (pH 8.3) was added to a separate well, and rBPI₂₃ at concentrations of 5 μ g/mL and 1 μ g/mL were also added as positive controls. Each plate was incubated at 37°C for 3 hours, and then 10 mL of molten overlay agarose (at approximately 45°C) was added into the level petri dish, allowed to harden and incubated overnight at 37°C. The next day, a clear zone was seen against the lawn of bacteria in those wells having bactericidal activity. In order to visually enhance this zone, a dilute Coomassie solution (consisting of 0.002% Coomassie Brilliant Blue, 27% methanol, 15% formaldehyde (37% stock solution) and water) was poured over the agar and allowed to stain for 24 hours. The bacterial zones were measured with a micrometer.

No bactericidal activity was discerned for the rBPI₂₃ fragments generated by CNBr or by Asp-N digestion, when tested at amounts up to 25 pmol/well. In contrast, this assay detected measurable bactericidal activity using rBPI₂₃ in amounts as low as 0.75 pmol/well. Reduced and alkylated rBPI₂₃, on the other hand, also was not bactericidal at amounts up to 100 pmol/well, while alkylated rBPI₂₃ retained bactericidal activity equivalent to rBPI₂₃.

EXAMPLE 3

Heparin Binding by BPI Proteolytic Fragments

5 rBPI₂₃ and the BPI proteolytic fragments produced according to Example 1 were evaluated in heparin binding assays according to the methods described in Example 1 in copending U.S. Patent Application Serial No. 08/093,202, filed July 15, 1993 and incorporated by reference. Briefly, each fragment was added to wells of a 96-well microtiter plate having a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) disposed at the bottom of the wells. Heparin binding of CNBr fragments was estimated using 100 picomoles of each fragment per well with a saturating concentration of ³H-heparin (20 µg/mL). Positive control wells contained varying amounts of rBPI₂₃. The wells were dried and subsequently blocked with a 0.1% bovine serum albumin (BSA) in phosphate buffered saline, pH 7.4 (blocking buffer). Dilutions of ³H-heparin (0.03-20 µCi/ml, avg. M.W. = 15,000; DuPont-NEN, Wilmington, DE) were made in the blocking buffer and incubated in the BPI peptide-containing wells for one hour at 4°C. The unbound heparin was aspirated and the wells were washed three times with blocking buffer, dried and removed for quantitation in a liquid scintillation counter (Model 1217, LKB, Gaithersburg, MD). Although BSA in the blocking buffer did show a low affinity and capacity to bind heparin, this was considered physiologically irrelevant and the background was routinely subtracted from the test compound signal. The specificity of fragment-heparin binding was established by showing that the binding of radiolabeled heparin was completely inhibited by a 100-fold excess of unlabeled heparin (data not shown).

25 The results, shown in Table II (as the mean values of duplicate wells ± the range between the two values), indicated that the CNBr fragments containing the amino acids 71-100 (C3) and 1-56 and 112-170 (C1,5) bound heparin to a similar extent. The CNBr fragment 171-196 also bound more heparin than the control protein (thaumatin, a protein of similar molecular weight and charge to rBPI₂₃).

30

The Asp-N fragments also demonstrated multiple heparin binding regions in rBPI₂₃. As seen in Table II, the 57-104 Asp-N fragment bound the highest amount of heparin, followed by the 1-38 and 116-193 fragments. These data, in combination with the CNBr fragment data, indicate that there are at least three separate heparin binding regions within rBPI₂₃, as demonstrated by chemically or enzymatically-generated fragments of rBPI₂₃, with the highest heparin binding capacity residing within residues 71-100.

TABLE II
Heparin Binding of rBPI₂₃ Fragments

<u>Fragments</u>	<u>Region</u>	<u>cpm³H-Heparin bound</u>
<u>CNBr Digest</u>		
C1,C5	1-56,112-170	82,918 ± 4,462
C2	57-70	6,262 ± 182
C3	71-100	81,655 ± 3,163
C4	101-111	4,686 ± 4
C6	171-196	26,204 ± 844
<hr/>		
<u>Asp-N Digest</u>		
A1	1-38	17,002 ± 479
A2	15-38	3,042 ± 162
A3	39-56	8,664 ± 128
A4	57-104	33,159 ± 1,095
A6a	116-193	13,419 ± 309
<hr/>		
rBPI ₂₃	1-193	51,222 ± 1,808
Thaumatococcus		7,432 ± 83
Wash Buffer		6,366 ± 46

EXAMPLE 4

Effect of BPI Proteolytic Fragments on an LAL Assay

BPI proteolytic fragments produced according to Example 1 were subjected to a *Limulus* Amoebocyte Lysate (LAL) inhibition assay to determine LPS binding properties of these fragments. Specifically, each of the fragments were mixed in Eppendorf tubes with a fixed concentration of *E. coli* 0113 LPS (4 ng/mL final concentration) and incubated at 37°C for 3 hours with

occasional shaking. Addition controls comprising rBPI₂₃ at 0.05 µg/mL were also tested. Following incubation, 360 µL of Dulbecco's phosphate buffered saline (D-PBS; Grand Island Biological Co. (GIBCO), Long Island, NY) were added per tube to obtain an LPS concentration of 200 pg/mL for the LAL assay. Each sample was then transferred into Immulon II strips (Dynatech, Chantilly, VA) in volumes of 50 µl per well.

Limulus amoebocyte Lysate (Quantitative Chromogenic LAL kit, Whitaker Bioproducts, Inc., Walkersville, MD) was added at 50 µL per well and the wells were incubated at room temperature for 25 minutes. Chromogenic substrate was then added at a volume of 100 µL per well and was well mixed. After incubation for 20 to 30 minutes at room temperature, the reaction was stopped with addition of 100 µL of 25% (v/v) acetic acid. Optical density at 405 nm was then measured in a multiplate reader (Model Vmax, Molecular Dynamics, Menlo Park, CA) with the results shown in Figure 2 in terms of percent inhibition of LPS. In this Figure, the filled circle represents rBPI₂₃; the open circle represents Asp-N fragment A3; the x represents Asp-N fragment A2; the filled square represents Asp-N fragment A4; the filled triangle represents Asp-N fragment A1A2; the open square represents Asp-N fragment A6a; the small open triangle represents CNBr fragment C3; and the small filled square represents CNBr fragment C1/C5.

The CNBr digest fraction containing amino acid fragments 1-56 and 112-170 inhibited the LPS-induced LAL reaction with an IC₅₀ of approximately 100 nM. This IC₅₀ is approximately 10-fold higher than the IC₅₀ for intact rBPI₂₃ (9 nM) in the same assay. The other CNBr digest fragments were found to be non-inhibitory.

A slightly different result was observed with fragments generated from the Asp-N digest, where three fragments were found to be inhibitory in the LAL assay. The fragment corresponding to amino acids 116-193 exhibited LAL inhibitory activity similar to intact rBPI₂₃ with complete inhibition of the LPS-induced LAL reaction at 15 nM. The fragments corresponding to amino acids 57-104 and 1-38 also inhibited the LAL assay, but required 10-fold

higher amounts. These results, in combination with the CNBr digest results, further supported the conclusion from previously-described experimental results that at least three regions of the rBPI₂₃ molecule have the ability to neutralize LBS activation of the LAL reaction, with the most potent region appearing to exist within the 116-193 amino acid fragment.

Immunoreactivity studies of the proteolytic fragments of rBPI₂₃ described in Example 1 were performed using ELISA assays. In such assays, a rabbit polyclonal anti-rBPI₂₃ antibody, capable of blocking rBPI₂₃ bactericidal and LAL inhibition properties, and two different, non-blocking mouse anti-rBPI₂₃ monoclonal antibodies were used to probe the rBPI₂₃ proteolytic fragments. The polyclonal antibody was found to be immunoreactive with the 116-193 and 57-104 Asp-N fragments and with the 1-56 and 112-170 CNBr fragments, while the murine monoclonal antibodies reacted only with an Asp-N fragment representing residues 1-14 of rBPI₂₃.

EXAMPLE 5

Preparation of 15-mer Peptides of BPI

In order to further assess the domains of biological activity detected in the BPI fragment assays described in Examples 1-4, 15-mer synthetic peptides comprised of 15 amino acids derived from the amino acid sequence of the 23kD amino terminal fragment of BPI were prepared and evaluated for heparin-binding activity, activity in a *Limulus* Amoebocyte Lysate Inhibition (LAL) assay and bactericidal activity. Specifically, a series of 47 synthetic peptides were prepared, in duplicate, each comprising 15 amino acids and synthesized so that each peptide shared overlapping amino acid sequence with the adjacent peptides of the series by 11 amino acids, based on the sequence of rBPI₂₃ as previously described in copending U.S. Patent Application Serial No. 08/093,202, filed July 15, 1993.

Peptides were simultaneously synthesized according to the methods of Maeji *et al.* (1990, *Immunol. Methods* 134: 23-33) and Gammon *et al.* (1991, *J. Exp. Med.* 173: 609-617), utilizing the solid-phase technology of Cambridge

Research Biochemicals Ltd. under license of Coselco Mimotopes Pty. Ltd. Briefly, the sequence of rBPI₂₃ (1-199) was divided into 47 different 15-mer peptides that progressed along the linear sequence of rBPI₂₃ by initiating a subsequent peptide every fifth amino acid. This peptide synthesis technology allows for the simultaneous small scale synthesis of multiple peptides on separate pins in a 96-well plate format. Thus, 94 individual pins were utilized for this synthesis and the remaining two pins (B,B) were subjected to the same steps as the other pins without the addition of activated FMOC-amino acids. Final cleavage of the 15-mer peptides from the solid-phase pin support employed an aqueous basic buffer (sodium carbonate, pH 8.3). The unique linkage to the pin undergoes a quantitative diketopiperazine cyclization under these conditions resulting in a cleaved peptide with a cyclo(lysylprolyl) moiety on the carboxyl-terminus of each peptide. The amino-termini were not acetylated so that the free amino group could potentially contribute to anion binding reactions. An average of about 15 µg of each 15-mer peptide was recovered per well.

EXAMPLE 6

Heparin Binding by 15-mer Peptides of BPI

The BPI 15-mer peptides described in Example 5 were subjected to a heparin binding assay according to the methods described in Example 3.

The results of these experiments are shown in Figure 3, expressed as the total number of cpm bound minus the cpm bound by control wells which received blocking buffer only. These results indicated the existence of three distinct subsets of heparin-binding peptides representing separate heparin-binding functional domains in the rBPI₂₃ sequence. In the BPI sequence, the first domain was found to extend from about amino acid 21 to about amino acid 55; the second domain was found to extend from about amino acid 65 to about amino acid 107; and the third domain was found to extend from about amino acid 137 to about amino acid 171. Material from the blank control pins showed no heparin binding effects.

EXAMPLE 7**Effect of 15-mer Peptides of BPI
on an *Limulus* Amoebocyte Lysate (LAL) Assay**

5 The 15-mer peptides described in Example 5 were assayed for LPS binding activity using the LAL assay described in Example 4.

The results of these experiments are shown in Figure 4. The data in Figure 4 indicated at least three major subsets of peptides representing three distinct domains of the rBPI₂₃ protein having LPS-binding activity resulting in significant LAL inhibition. The first domain was found to extend from about
10 amino acid 17 to about amino acid 55; the second domain was found to extend from about amino acid 73 to about amino acid 99; and the third domain was found to extend from about amino acid 137 to about amino acid 163. In addition, other individual peptides also exhibited LAL inhibition, as shown in the Figure. In contrast, material from blank control pins did not exhibit any
15 LPS neutralizing effects as measured by the LAL assay.

EXAMPLE 8**Bactericidal Effects of 15-mer Peptides of BPI**

20 The 15-mer peptides described in Example 5 were tested for bactericidal effects against the rough mutant strain of *E. coli* bacteria (J5) in a radial diffusion assay as described in Example 2. Products from the blank pins (B, B) were tested as negative controls.

The results of the assay are shown in Figure 5. The only 15-mer peptide found to have bactericidal activity was a peptide corresponding to
25 amino acids 85-99 of the BPI protein. As is seen in Figure 5, the positive control wells having varying amounts of rBPI₂₃ also showed bactericidal activity, while the buffer and blank pin controls did not.

The results of these bactericidal assays, along with the heparin binding and LAL assays described in the above Examples, indicate that there exist
30 discrete functional domains in the BPI protein.

The results shown in Examples 1-8 above indicate that rBPI₂₃ contains at least three functional domains that contribute to the total biological activity

of the molecule. The first domain appears in the sequence of amino acids between about 17 and 45 and is destroyed by Asp-N cleavage at residue 38. This domain is moderately active in both the inhibition of LPS-induced LAL activity and heparin binding assays. The second functional domain appears in the region of amino acids between about 65 and 99 and its inhibition of LPS-induced LAL activity is diminished by CNBr cleavage at residue 70. This domain also exhibits the highest heparin binding capacity and contains the bactericidal peptide, 85-99. The third functional domain, between about amino acids 142 and 169, is active in the inhibition of LPS-induced LAL stimulation assay and exhibits the lowest heparin binding capacity of the three regions.

EXAMPLE 9

Preparation of BPI Individual Functional Domain Peptides

Based on the results of testing the series of overlapping peptides described in Examples 5 through 8, BPI functional domain peptides from each of the functionally-defined domains of the BPI protein were prepared by solid phase peptide synthesis according to the methods of Merrifield, 1963, *J. Am. Chem. Soc.* 85: 2149 and Merrifield *et al.*, 1966, *Anal. Chem.* 38: 1905-1914 using an Applied Biosystems, Inc. Model 432 peptide synthesizer. BPI functional domain peptides were prepared having the amino acid sequences of portions of amino acid residues 1-199 of BPI as set out in Table III below and designated BPI.2 through BPI.5 and BPI.8.

TABLE III
BPI Individual Functional Domain Peptides

	Polypeptide No.	Domain	Amino Acid Region	Amino Acid Residues	MW (daltons)
5	BPI.2	II	85-99	15	1828.16
	BPI.3	II	73-99	27	3072.77
	BPI.4	I	25-46	22	2696.51
	BPI.5	III	142-163	22	2621.52
	BPI.8	II	90-99	10	1316.8

10

EXAMPLE 10**Heparin Binding Activity by BPI Individual Functional Domain Peptides**

BPI individual functional domain peptides BPI.2, BPI.3, and BPI.8, along with rBPI₂₁Δcys were assayed for heparin binding activity according to the methods described in Example 3. The results are shown in Figure 6 and indicate that BPI.3 and rBPI₂₁Δcys had moderate heparin binding activity and BPI.2 and BPI.8 had little or no heparin binding activity.

15

EXAMPLE 11

Heparin Neutralization Activity of BPI Individual Functional Domain Peptides

20

BPI functional domain peptides BPI.2, BPI.3, BPI.4, BPI.5, BPI.6, and BPI.8, along with rBPI₂₃ as a positive control, were assayed for their effect on thrombin inactivation by ATIII/heparin complexes according to the method of Example 3 in copending and co-assigned U.S. Patent Application Serial No. 08/093,202, filed July 15, 1993, incorporated by reference. Specifically, a Chromostrate™ anti-thrombin assay kit (Organon Teknika Corp., Durham, NC) was used to examine the inhibition of purified thrombin by preformed ATIII/heparin complexes in plasma.

25

Briefly, the assay was performed in 96 well microtiter plates in triplicate with a final volume per well of 200 μL. Varying concentrations of

30

the BPI functional domain peptides ranging from 1.0 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ were assayed to determine their effect on thrombin inhibition in the presence of pre-formed ATIII/heparin complexes. The order of addition of assay components was as follows: 1) a dilution series of rBPI₂₃ or BPI functional domain peptides or thaumatin as a control protein, with final concentrations of 100, 50, 25, 10 and 1 $\mu\text{g/well}$, diluted in PBS in a final volume of 50 μL ; 2) 50 μL plasma diluted 1:100 in a buffer supplied by the manufacturer; 3) 50 μL thrombin at 1 nKat/mL in a buffer supplied by the manufacturer; and 4) 50 μL chromogenic substrate at a concentration of 1 $\mu\text{mol/mL}$ in water. The reaction was allowed to proceed for 10 minutes at 37°C and stopped with the addition of 50 μL 0.1M citric acid. The colorimetric reaction was quantitated on a microplate reader as described in Example 3.

The results of these assays are shown in Figures 7a and 7b, which depict the sample concentrations as weight or molar concentrations respectively. BPI functional domain peptides BPI.3 and BPI.5 each had the most significant heparin neutralization effects. In these assays, the control protein, thaumatin, showed no neutralizing effect and was essentially equivalent to the buffer control at all protein concentrations.

EXAMPLE 12

LPS Neutralization Activity by LAL Assay of BPI Individual Functional Domain Peptides

BPI functional domain peptides BPI.2, BPI.3, and BPI.8, along with rBPI₂₃ as a positive control, were evaluated in the LAL assay according to the method of Example 4 herein to determine LPS binding and inhibition properties of these peptides. The experiments were performed essentially as described in Example 3 and the results are shown in Figures 8a and 8b, which depict the sample concentrations as weight or molar concentrations respectively. The results showed that BPI.3 had moderate LPS inhibition activity and that BPI.2 and BPI.8 had no significant LPS inhibition activity.

EXAMPLE 13**Bactericidal Activity Assay of BPI Individual
Functional Domain Peptides**

5 BPI functional domain peptides BPI.2, BPI.3, and BPI.8, along with
rBPI₂₃ as a positive control, were tested for bactericidal effects against *E. coli*
J5 (rough) and *E. coli* 0111:B4 (smooth) bacteria in a radial diffusion assay
according to the methods of Example 2. The results of these assays are
depicted in Figures 9a-9d. These results demonstrated that each of the BPI
functional domain peptides BPI.2 and BPI.3 exhibited bactericidal activity while
10 BPI.8 had little to no bactericidal activity. Each of the bactericidal peptides
showing bactericidal activity tended to be more effective against the rough than
the smooth *E. coli* strain.

In additional experiments, broth antibacterial assays were conducted to
further determine the bactericidal activity of certain of the BPI peptides.
15 Specifically, either *E. coli* J5 (rough) or *E. coli* 0111:B4 (smooth) bacteria
were selected from single colonies on agar plates and used to inoculate 5 mL
of Mueller Hinton broth and incubated overnight at 37°C with shaking. The
overnight culture was diluted (~ 1:50) into 5 mL fresh broth and incubated
at 37°C to log phase (~ 3 hours). Bacteria were pelleted for 5 minutes at
20 3000 rpm (1500 x g). Bacterial pellets were resuspended in 5 mL PBS and
diluted to 2×10^6 cells/mL in the Mueller Hinton broth (wherein 1 OD₅₇₀ unit
equals 1.25×10^9 CFU/mL). The BPI functional domain peptides to be tested
were diluted to 200 µg/mL in broth and serially diluted 2-fold in 96 well
culture plates (100 µL volume). All items were at 2-fold final concentration
25 and experiments were conducted in triplicate. Bacteria were added at 100
µL/well and the plates were incubated on a shaker at 37°C for a 20 hour
period. The plates were then read on an ELISA plate multiple reader at 590
nm. One of the triplicate wells from each peptide concentration was selected
for colony forming unit (CFU) determination. A 30 µL aliquot was added to
30 270 µL of PBS and further ten-fold serial dilutions were performed. Then a
50 µL aliquot was plated on tryptic soy agar and incubated overnight.
Colonies were counted and final bacterial concentrations determined. The

results of these assays are depicted in Figures 9e (for *E. coli* J5) and 9f (for *E. coli* 0111:B4). As shown in these Figures, BPI functional domain peptide BPI.3 had significant anti-bacterial activity against *E. coli* J5 bacteria and less activity against *E. coli* 0111:B4 bacteria.

5

EXAMPLE 14

Preparation of BPI Combination Functional Domain Peptides

Combination peptides were prepared using solid-phase chemistry as described in Example 9. The sequences of these peptides are shown in Table IV. It will be noted that the peptides designated BPI.7, BPI.9 and BPI.10 represent partial or even multiple repeats of certain BPI sequences. Specifically, BPI.7 comprises a 20-mer consisting of amino acid residues 90-99 repeated twice in a single linear peptide chain. BPI.10 comprises an approximately 50:50 admixture of a 25-mer (designated BPI.10.1; SEQ ID NO:55) and a 26-mer (designated BPI.10.2; SEQ ID NO:65) consisting of amino acid residues 94-99, 90-99, 90-99 and 93-99, 90-99, 90-99, respectively, in a single linear peptide chain. BPI.9 comprises a 16-mer comprising amino acid residues 94-99 followed by residues 90-99 in a single linear peptide chain.

These peptides were used in each of the BPI activity assays described in Examples 10-13 above. In the heparin binding assay described in Example 10 and shown in Figure 6, BPI.7 had extremely high heparin binding capacity. In the heparin neutralization assay described in Example 11 and shown in Figures 7a and 7b, BPI.7 had significant heparin neutralization effects compared with rBPI₂₃. In the LAL assay described in Example 12 and shown in Figures 8a and 8b, BPI.7 had significant LPS inhibition properties. In bactericidal assays using radial diffusion plates as described in Example 13 and shown in Figures 9a-9d, each of the BPI functional domain peptides BPI.7, BPI.9 and BPI.10.1 and BPI.10.2 exhibited bactericidal activity, and significant bactericidal activity was also found for BPI.7, BPI.9 and BPI.10.1 and BPI.10.2 against both rough and smooth variant strains of *E.coli* in broth

assays. The BPI.10 peptides exhibited the highest bactericidal activity observed against either bacterial strain.

These bactericidal activity results obtained with peptides BPI.7 and BPI.10 showed that a linear dimer (BPI.7) and a mixture of linear multimers (BPI.10.1 and BPI.10.2) of the BPI domain II peptide KWKAQKRFLK (*i.e.*, BPI.8, SEQ ID NO:8) had bactericidal activity against *E. coli* strain J5, and that the monomer (BPI.8) showed essentially no bactericidal activity. Moreover, both the dimer and the multimer peptides had higher bactericidal activity than that of BPI.9, comprising amino acids 94-99, 90-99. On the basis of these results, the additional peptides shown in Table IV were synthesized using the methods described in Example 9.

TABLE IV
BPI Combination Functional Domain Peptides

	BPI peptide No.	Amino Acid Region	Amino Acid Residues	MW (daltons)
15	BPI.7	90-99, 90-99	20	2644.66
	BPI.8	90-99	10	1316.8
	BPI.9	94-99, 90-99	16	2131.34
	BPI.10.1	94-99, 90-99, 90-99	25	3319.19
20	BPI.10.2	93-99, 90-99, 90-99	26	3447.32
	BPI.13	148-161	14	1710.05
	BPI.29	148-161, 148-161	28	3403.1
	BPI.30	90-99, 148-161	24	3023.86
	BPI.63	85-99, 148-161	29	3524.4

EXAMPLE 15

Bactericidal Activity of Combination Functional Domain Peptides

The BPI combination functional domain peptides described in Example 14 were used in radial diffusion bactericidal assays essentially as described in Examples 2 and 13 above. These results are shown in Figures 10a-10e. The

results shown in Figure 10a demonstrate that BPI.8, comprising one copy of a domain II peptide (amino acids 90-99), had no detectable bactericidal activity against *E. coli* J5 cells at concentrations of 1000 $\mu\text{g/mL}$. In contrast, BPI.13, comprising one copy of a domain III monomer (amino acids 148-161) showed appreciable bactericidal activity at concentrations greater than 30 $\mu\text{g/mL}$. BPI.29, comprising two copies of a domain III monomer BPI.13, had greater bactericidal activity, and BPI.30, comprising a linear combination of the domain II peptide BPI.8 and the domain III peptide BPI.13, showed the highest bactericidal activity against J5 cells, approximating that of BPI.

Figure 10b shows the results of experiments with domain II peptides comprising BPI.8, BPI.7 and BPI.10. (See also summary Table VIII.) Although BPI.8 showed no bactericidal activity against *E. coli* J5 cells at concentrations of 1000 $\mu\text{g/mL}$, the combination peptides BPI.7 and BPI.10 showed high levels of bactericidal activity.

Additional experiments were performed using various other bacteria as target cells to examine the range of bactericidal killing of these BPI functional domain peptides. Figure 10c shows the results of radial diffusion experiments using *E. coli* strain O7-K1. In these experiments, rBPI₂₃ showed no bactericidal activity at concentrations of 100 $\mu\text{g/mL}$, and low bactericidal activity even at concentrations of 1000 $\mu\text{g/mL}$. Similarly low levels of bactericidal activity were found with the peptides BPI.8 comprising the domain II (DII) monomer and BPI.13 comprising the domain III (DIII) monomer, although the amount of activity of BPI.13 was found to be higher than that of rBPI₂₃. Surprisingly, the domain II dimer BPI.7 and the domain II-domain III (DII-DIII) heterodimer BPI.30 showed high levels of bactericidal activity, and the domain III dimer BPI.29 showed moderate bactericidal activity. These results demonstrated that peptides of the functional BPI functional domain identified herein possess bactericidal activity qualitatively different from the bactericidal activity of the BPI molecule itself.

Figures 10d and 10e show results that further demonstrate that the homo- and heterodimers described herein have qualitatively and quantitatively

different bactericidal activity spectra of susceptible bacteria. Figure 10d shows the results of radial diffusion assays using *Klebsiella pneumoniae* bacteria. The DII-DIII heterodimer BPI.30 showed the highest amount of bactericidal activity against this bacteria, the DIII homodimer BPI.29 showed moderate levels of activity, and the DII dimer (BPI.7) and DIII monomer (BPI.13) showed low levels of activity. BPI.8, comprising the DII monomer, showed no bactericidal activity at concentrations of 800 $\mu\text{g/mL}$, consistent with the lack of bactericidal activity of this peptide seen with the *E. coli* strains tested.

Figure 10e shows the levels of bactericidal activity found in radial diffusion experiments using the Gram-positive bacterium *Staphylococcus aureus*. The DII-DIII heterodimer BPI.30 showed the highest amount of bactericidal activity against this bacteria, the DIII homodimer BPI.29 showed moderate levels of activity, and DII dimer (BPI.7) and the DIII monomer (BPI.13) showed low levels of activity. BPI.8, comprising the DII monomer, showed no bactericidal activity at concentrations of 800 $\mu\text{g/mL}$, consistent with the lack of bactericidal activity of this peptide seen with the other bacteria.

These results showed that the homo- and heterodimers disclosed herein possessed varying amounts of bactericidal activity, which varied both with regard to the amount of such activity and the minimum effective concentration of the peptide necessary for bactericidal activity to be detected. These results also showed that these peptides possessed quantitatively and, more surprisingly, qualitatively different bactericidal activity than the BPI itself.

EXAMPLE 16

Additional Bactericidal Activity of BPI Combination Functional Domain Peptides

In light of the results of the experiments disclosed in Example 15, the bactericidal activity of domain II-domain III combination peptides were compared with the bactericidal activity of each of the component BPI domain II and domain III peptides, against a number of different bacteria and other microorganisms. The following BPI functional domain peptides as described above were used in radial diffusion bactericidal assays (Example 2) and broth

bactericidal assays (Example 13) essentially as described in Example 15 above. These results are shown in Figures 11a-11q. These Figures show results of bactericidal assays using the following bacterial strains:

5	<u>Gram-negative bacteria</u>	<u>BPI peptides tested</u>
	<i>Pseudomonas aeruginosa</i>	BPI.8, BPI.13, BPI.30
	<i>E. coli</i> O18:K1:H7	BPI.8, BPI.13, BPI.30
10	<i>Klebsiella pneumoniae</i>	BPI.8, BPI.13, BPI.30
	<i>E. coli</i> O75	BPI.8, BPI.13, BPI.30
	<i>Serratia marcescens</i>	BPI.8, BPI.13, BPI.30
	<i>Proteus mirabilis</i>	BPI.2, BPI.13, BPI.30
	<i>Salmonella typhurium</i>	BPI.23, BPI.30
15	<i>E. coli</i> O86a:K61	BPI.23, BPI.30
	<i>E. coli</i> O4:K12	BPI.30
	<u>Gram-positive bacteria</u>	
20	<i>Streptococcus pneumonia</i>	BPI.29, BPI.30., BPI.48, BPI.55, BPI.13, BPI.69
	<i>Bacillus megaterium</i>	BPI.2, BPI.7, BPI.45, BPI.46, BPI.47, BPI.48
25	<i>Staphylococcus aureus</i>	BPI.7, BPI.8, BPI.10, BPI.13, BPI.30
	<u>Fungi</u>	
	<i>Candida albicans</i>	BPI.30, BPI.13, BPI.29, BPI.48, BPI.2
30	The results of these experiments are summarized as follows. None of the BPI peptides tested showed any bactericidal activity against <i>S. marcescens</i> (Figure 11f) or <i>P. mirabilis</i> (Figure 11g). BPI.8 showed no bactericidal activity against any organism tested at concentrations up to about 2000 pmol. BPI.13 and BPI.30 showed bactericidal activity against <i>P. aeruginosa</i> (Figure 11a), <i>E. coli</i> O18:K1:H7 (Figure 11b), <i>K. pneumoniae</i> (Figure 11c), and <i>E. coli</i> O75 (Figure 11d). Additionally, BPI.30 showed bactericidal activity against <i>S. typhurium</i> (Figure 11h), and, in broth assays, <i>E. coli</i> O86a:K51 (Figure 11j) and <i>E. coli</i> O4:K12 (Figure 11k). BPI.23 showed bactericidal activity in a radial diffusion assay against <i>E. coli</i> O86a:K61 (Figure 11i).	
35		

Additionally, BPI.30 showed bactericidal activity against *E. coli* O86a:K61 in human serum (Figure 11l).

The bactericidal capacity of BPI peptides provided by the invention was also tested against Gram-positive bacteria. Surprisingly, every BPI peptide tested showed some bactericidal activity in radial diffusion assays using *S. aureus* (Figure 11e), *S. pneumoniae* (Figure 11m) and *B. megaterium* (Figure 11n) at amounts ranging between about 20 and about 2000 pmol. These results compared favorably with bactericidal activity of the antibiotics gentamicin and vancomycin (Figure 11o).

Most surprisingly, one peptide, BPI.13, was found to have fungicidal activity in a broth assay using *C. albicans* (Figures 11p and 11q). As shown in these Figures, the activity of BPI.13 is clearly distinguishable from the much lower activity levels of BPI.2, BPI.29, BPI.30, and BPI.48. These results demonstrate that the BPI functional domain peptides of the invention have antimicrobial activity qualitatively distinct from the activity previously reported for native BPI.

EXAMPLE 17

Heparin Neutralization Activity of BPI Combination Functional Domain Peptides

The *in vitro* and *in vivo* heparin neutralization capacity of the BPI combination functional domain peptides prepared in Example 14 was determined by assaying the ability of these peptides to counteract the inhibitory effect of heparin on clotting time of heparinized blood and plasma.

In vitro, the effect of BPI combination functional domain peptides was determined on heparin-mediated lengthening of activated partial thrombin time (APTT). The APTT is lengthened by the presence of endogenous or exogenous inhibitors of thrombin formation, such as therapeutically administered heparin. Thus, agents which neutralize the anti-coagulant effects of heparin will reduce the APTT measured by the test. Citrated human plasma (200 μ L) was incubated for 1 minute at 37°C with either 15 μ L of diluent (0.15 M

NaCl, 0.1 M Tris-HCl, pH 7.4) or 15 μ L of the diluent also containing 25 μ g/mL heparin (187 units/mg). Various concentrations (from 0.0 to 56 μ g/mL) of rBPI₂₃, rBPI₂₁ Δ cys, or BPI combination peptides BPI.29 (the DIII homodimer) and BPI.30 (heterodimer DII + DIII) in a volume of 15 μ L were added, followed immediately by 100 μ L of thrombin reagent (Catalog No. 845-4, Sigma Chemical Co., St. Louis, MO). Clotting time (thrombin time) was measured using a BBL Fibrometer (Becton Dickinson Microbiology Systems, Cockeysville, MD). The results are shown in Figures 12a, 12b and 12e. Figure 12a shows the relative decrease caused by addition of varying amounts of rBPI₂₃ or rBPI₂₁ Δ cys to the heparin-prolonged APTT. These results establish that each of these BPI-related proteins inhibits the heparin-mediated lengthening of APTT. Figure 12b shows that the BPI combination peptides BPI.29 and BPI.30 also inhibit the heparin-mediated lengthening of APTT. Figure 12e illustrates the results obtained with BPI.30 on a non-log scale. Figure 12g shows that BPI.29, BPI.30, and BPI.7 have the greatest effect on the clotting time of heparinized blood in the assay. BPI.3 and rBPI₂₃ show a smaller effect, and BPI.14, BPI.2, BPI.4, BPI.5, BPI.7, and rLBP₂₅, rBPI and rBPI₂₁ Δ cys all show less of a decrease in clotting times of heparinized blood in this assay.

The *in vivo* effect of exemplary BPI combination peptides on APTT in heparinized rats was determined and compared with the *in vivo* effect of rBPI₂₃. APTT is lengthened by the presence of endogenous or exogenous inhibitors of thrombin formation, such as therapeutically administered heparin. Agents which neutralize the anti-coagulant effects of heparin will reduce the APTT as measured by this test. Sprague-Dawley rats housed under NIH guidelines were administered with 100 U/kg heparin by bolus intravenous injections *via* the animals' tail vein followed 5 minutes later by administration of varying amounts of test or control protein as compared with rBPI₂₃. The APTT was then determined from blood samples collected from the abdominal aorta 2 minutes after the administration of the test or control protein. The APTT of untreated animals, as well as animals treated only with a BPI peptide, was also

determined. Figure 12c shows the dose dependence of rBPI₂₃ inhibition of heparin-mediated lengthening of partial thromboplastin time, and that administration of about 5mg/kg results in a APTT of the heparinized and BPI-treated animals that is almost the same as the untreated control animals. The results of similar experiments shown in Figure 12d demonstrate that the unrelated protein thaumatin has no effect on APTT times in heparinized animals. The administration of BPI.10 peptide results in a APTT in heparinized animals that is essentially the same as the APTT in control animals treated with BPI.10 alone. Similar results using BPI.30 were also obtained (Figure 12f).

These results show that BPI functional domain combination peptides (e.g., BPI.10 and BPI.30) and rBPI₂₃ effectively neutralize heparin inhibition of coagulation proteases. Based on these characteristics, BPI combination functional domain peptides of the invention are projected to be useful in the clinical neutralization of heparin anti-coagulant effects in dosages generally corresponding functionally to those recommended for protamine sulfate, but are not expected to possess the severe hypotensive and anaphylactoid effects of that material.

EXAMPLE 18

Preparation and Functional Activity Analysis of BPI Substitution Variant Functional Domain Peptides

The results obtained above with peptides from functional domains II and III prompted a further effort to determine the functionally-important amino acid residues within these peptides. Accordingly, a series of peptides comprising the amino acid sequences of domains II and III were prepared in which one of the amino acids in the sequence was substituted with an alanine residue. Diagrams of the domain peptides used in the substitution experiments are shown in Figure 13 (domain II; IKISGKWKAQKRFLK, SEQ ID No.:7) and Figure 14 (domain III; KSKVGWLIQLFHKK, SEQ ID No.:13). These peptide series were then tested for heparin binding affinity (K_d), heparin binding capacity (Hep-CAP), LPS neutralization as determined using the *Limulus*

Ameboctye Lysate assay (LAL), and bactericidal activity against *E. coli* J5 using the radial diffusion assay (RAD), each assay as performed as described in the Examples above.

5 The results, shown in Table V (domain II) and Table VI (domain III), are expressed in terms of the fold difference in activity in each of these assays (except for the LAL assay where relative differences are noted) between the BPI functional domain II and domain III peptides and each alanine substituted variant peptide thereof.

10 For domain II peptides, most alanine-substituted peptides showed an approximately 2- to 10-fold reduction in bactericidal activity in the radial diffusion assay. Exceptions to this overall pattern include BPI.19 (Gly₈₉ → Ala₈₉), BPI.22 (Lys₉₂ → Ala₉₂), BPI.23 (Gln₉₄ → Ala₉₄) and BPI.24 (Lys₉₅ → Ala₉₅). In contrast, most alanine-substituted peptides showed no difference in the LAL assay; BPI.17 (Ile₈₇ → Ala₈₇) and BPI.21 (Trp₉₁ → Ala₉₁) showed a
15 moderate and large decrease in activity, respectively, in this assay. For BPI.21, these results were consistent with the more than 10-fold reduction in bactericidal activity found for this peptide, indicating that amino acid 91 (a tryptophan residue in the native sequence) may be particularly important in conferring biological activity on the peptide.

20 The effect of alanine substitution on heparin binding and capacity was, in almost all cases, no more than 2-fold more or less than the unsubstituted peptide. One exception was the heparin binding capacity of BPI.21, which was 4-fold lower than the unsubstituted peptide. This further supports the earlier results on the particular sensitivity of the various activities of these peptides
25 to substitution at Trp₉₁. In most cases, the effect on both the K_d of heparin binding and heparin binding capacity was consistent and of about the same magnitude. In some instances, the heparin binding capacity of the substituted peptide decreased, although the K_d increased slightly (BPI.18; Ser₈₈ → Ala₈₈), or decreased slightly (BPI.24). There were also instances where capacity was
30 unchanged even though the K_d increased (BPI.20; Lys₉₀ → Ala₉₀) or decreased

(BPI.19). In one instance the affinity remained unaffected and the capacity decreased almost 2-fold (BPI.25; Arg₉₆ → Ala₉₆).

5 These results indicated the existence of at least one critical residue in the domain II sequence (Trp₉₁), and that the activities of the domain II peptides were for the most part only minimally affected by alanine substitution of the other domain II amino acid residues.

10 For domain III peptides, most alanine-substituted peptides showed an approximately 2- to 5-fold reduction in bactericidal activity in the radial diffusion assay. Exceptions to this overall pattern include BPI.35 (Gly₁₅₂ → Ala₁₅₂), BPI.39 (Gln₁₅₆ → Ala₁₅₆), BPI.42 (His₁₅₉ → Ala₁₅₉) and BPI.44 (Lys₁₆₁ → Ala₁₆₁). Most alanine-substituted peptides showed no difference in the LAL assay; BPI.31 (Lys₁₄₈ → Ala₁₄₈), BPI.32 (Ser₁₄₉ → Ala₁₄₉), BPI.33 (Lys₁₅₀ → Ala₁₅₀), and BPI.34 (Val₁₅₁ → Ala₁₅₁) showed a moderate decrease in LPS-binding activity, and BPI.36 (Trp₁₅₃ → Ala₁₅₃) and BPI.40 (Leu₁₅₇ → Ala₁₅₇)
15 showed a large decrease in LPS-binding activity in this assay. For both BPI.36 and BPI.40, these results were consistent with the approximately 5-fold reduction in bactericidal activity found for these peptides, indicating that the hydrophobic amino acids Trp₁₅₃ and Leu₁₅₇ in the native sequence may be particularly important in conferring biological activity on the peptide.

20 Effects of alanine substitution on heparin binding and capacity were of similar magnitude, being no more than about 5-fold more or less than the unsubstituted peptide. In almost every case, the type of effect of alanine substitutions on both the K_d of heparin binding and heparin binding capacity was consistent and of about the same magnitude, unlike the findings with the
25 domain II alanine substitution peptides. In one instance (BPI.42; His₁₅₉ → Ala₁₅₉), the heparin binding capacity was unaffected although the K_d declined slightly (1.2-fold). In only one instance was the K_d of heparin binding and heparin capacity increased slightly (BPI.35; Gly₁₅₂ → Ala₁₅₃); an increase of only 10% was found.

30 Like the results found with the domain II alanine-substitution peptides, these results indicated the existence of at least one critical residue in the

domain III sequence (Trp₁₅₃), and possibly at least one other (Leu₁₅₇). The results also showed that, unlike the domain II alanine-substituted peptides, almost one-half of the substitutions resulted in at least a 2-fold difference in the activities tested. In 6 cases, all four of the tested activities decreased, and
5 in 10 instances bactericidal activity, the K_d of heparin binding and heparin capacity decreased. In only one instance (BPI.35, Gly₁₅₂ → Ala₁₅₂) was the activity in the bactericidal, heparin binding K_d and heparin capacity assays found to have increased, albeit slightly.

10 These results indicate that alanine replacement of the hydrophobic amino acid residues Trp₉₁, Trp₁₅₃ and Leu₁₅₇ have the greatest effect on the activities of these BPI functional domain substitution peptides. This result is unexpected in light of the cationic nature of rBPI₂₃. In fact, domain II alanine substitution peptides in which lysine is replaced either by alanine or phenylalanine showed dramatic increases in activity (*e.g.*, BPI.24, BPI.73).

TABLE V
BPI DOMAIN II ALANINE SUBSTITUTION PEPTIDES

		<u>FOLD CHANGE IN ACTIVITY</u>			
		<u>RAD</u>	<u>LAL</u>	<u>HEPK₂</u>	<u>HEPCAP</u>
BPI.2	I K I S G K W K A Q K R F L K	↓2.2	=	↓1.1	↓1.4
BPI.15	A	↓1.8	=	↓1.5	↓1.6
BPI.16	A	↓4.5	↓	↓1.3	↓1.8
BPI.17	A	↓1.6	=	↓1.1	↓1.3
BPI.18	A	↓1.4	=	↓1.3	=1.0
BPI.19	A	↓1.1	=	↓1.4	=1.0
BPI.20	A	↓10.4	↓↓	↓1.5	↓4.0
BPI.21	A	=1.0	=	↓1.1	↓1.5
BPI.22	A	↓2.2	=	↓2.0	↓1.4
BPI.23	A	↓3.8	=	↓2.1	↓2.1
BPI.24	A	↓3.8	=	=1.0	↓1.9
BPI.25	A	↓4.0	=	↓1.5	↓1.8
BPI.26	A	↓2.5	=	↓1.7	↓1.7
BPI.27	A	↓2.4	=	↓1.3	↓1.3
BPI.28	A				

5

10

15

20

TABLE VI
BPI DOMAIN III ALANINE SUBSTITUTION PEPTIDES
FOLD REDUCTION IN ACTIVITY

				<u>RAD</u>	<u>LAL</u>	<u>HEPK_d</u>	<u>HEPCAP</u>
5	BPI.13	K S K V G W L I Q L F H K K					
	BPI.31	A		↓2.3	↓	↓3.9	↓1.8
	BPI.32	A		↓1.5	↓	↓2.9	↓1.7
	BPI.33	A		↓1.4	↓	↓2.0	↓1.6
10	BPI.34	A		↓2.2	↓	↓1.9	↓1.8
	BPI.35	A		↓1.5	=	↓1.1	↓1.1
	BPI.36	A		↓4.9	↓↓	↓2.6	↓4.6
	BPI.37	A		↓3.8	=	↓5.2	↓2.7
	BPI.38	A		↓5.0	=	↓1.7	↓1.9
15	BPI.39	A		↓1.1	=	↓1.3	↓1.1
	BPI.40	A		↓5.2	↓↓	↓1.8	↓2.3
	BPI.41	A		↓4.3	=	↓2.1	↓3.1
	BPI.42	A		↓2.2	=	↓1.2	=1.0
	BPI.43	A		↓1.3	=	↓2.0	↓1.3
20	BPI.44			↓1.2	=	↓1.7	↓1.1

EXAMPLE 19**Summary of Biological Activity of BPI Functional Domain Peptides**

The distribution of the peptides into construct categories is presented in Table VII below.

5 The BPI functional domain peptides of this invention, or representative subsets thereof, have been assayed for the following biological activities: bactericidal activity against Gram-negative and Gram-positive bacteria, and against certain other microorganisms; LPS binding and neutralization activities; and heparin binding and heparin neutralization activities.

10 BPI functional domain peptides were assayed for bactericidal activity on *E. coli* J5 bacteria and for heparin binding as described in Examples 8 and 6, respectively. The assay results for exemplary peptides of the present invention are summarized in Table VIII for the Gram-negative bacteria *E. coli* J5 (rough) and *E. coli* O113 (smooth) and the Gram-positive bacteria *S. aureus*. The bactericidal
15 activities are expressed as the amount of peptide (pmol/well and $\mu\text{g/well}$) required to generate a 30 mm² bactericidal zone.

TABLE VII

	<u>BPI Peptide</u>	<u>Seq ID No.</u>	<u>Peptide Sequence</u>
5	I. BPI individual functional domain peptides		
	Domain I		
	<u>Peptides</u>		
	BPI.1	4	QQGTAALQKELKRIK
10	BPI.4	3	LQKELKRIKIPDYSDFSFKIKHL
	BPI.14	2	GTAALQKELKRIKIPDYSDFSFKIKHLGKGH
	BPI.54	5	GTAALQKELKRIKIP
	Domain II		
	<u>Peptides</u>		
15	BPI.2	7	IKISGKWKAQKRFLK
	BPI.3	11	NVGLKFSISNANIKISGKWKAQKRFLK
	BPI.8	8	KWKAQKRFLK
	Domain III		
	<u>Peptides</u>		
20	BPI.5	67	VHVVHISKSKVGWLIQLFHKKIE
	BPI.11	13	KSKVWLIQLFHKK
	BPI.12	14	SVHVHISKSKVGWLIQLFHKKIESALRNK
	BPI.13	15	KSKVGWLIQLFHKK
25	BPI.55	61	GWLIQLFHKKIESALRNKMNS
	II. Linear and branched-chain combination peptides		
	Domain II		
	<u>Peptides</u>		
	BPI.7	54	KWKAQKRFLKKWKAQKRFLK
30	BPI.9	51	KRFLKKWKAQKRFLK
	BPI.10.1	55	KRFLKKWKAQKRFLKKWKAQKRFLK
	BPI.10.2	65	QKRFLKKWKAQKRFLKKWKAQKRFLK
	MAP.1		β -Ala-N α ,N ϵ -[N α ,N ϵ (BPI.2)Lys]Lys

TABLE VII (cont'd.)

	BPI Peptide	Seq ID No.	Peptide Sequence
5			
	II. Linear and branched-chain combination peptides (cont'd.)		
	Domain III <u>Peptides</u>		
10	BPI.29	56	KSKVGWLIQLFHKKKSKVGWLIQLFHKK
	MAP.2		β -Ala-N α ,Ne-[N α ,Ne(BPI.13)Lys]Lys
	III. Single amino acid substitution peptides		
	Domain II <u>Peptides</u>		
15	BPI.15	16	AKISGKWKAQKRFLK
	BPI.16	17	IAISGKWKAQKRFLK
	BPI.17	18	IKASGKWKAQKRFLK
	BPI.18	19	IKIAGKWKAQKRFLK
	BPI.19	20	IKISAKWKAQKRFLK
20	BPI.20	21	IKISGAWKAQKRFLK
	BPI.21	22	IKISGKAKAQKRFLK
	BPI.22	23	IKISGKWAAQKRFLK
	BPI.23	24	IKISGKWKAQKRFLK
	BPI.24	25	IKISGKWKAQARFLK
25	BPI.25	26	IKISGKWKAQKAFLK
	BPI.26	27	IKISGKWKAQKRALK
	BPI.27	28	IKISGKWKAQKRFAK
	BPI.28	29	IKISGKWKAQKRFLA
	BPI.61	48	IKISGKFKAQKRFLK
30	BPI.73	62	IKISGKWKAQFRFLK
	BPI.77	72	IKISGKWKAQWRFLK
	BPI.79	73	IKISGKWKAQKRFLK
	BPI.81	75	IKISGKWKAQKRFLK

TABLE VII (cont'd.)

	<u>BPI</u> <u>Peptide</u>	Seq ID No.	<u>Peptide</u> <u>Sequence</u>
5			
	III. Single amino acid substitution peptides (cont'd.)		
	Domain III		
	<u>Peptides</u>		
	BPI.31	33	ASKVGWLIQLFHKK
10	BPI.32	34	KAKVGWLIQLFHKK
	BPI.33	35	KSAVGWLIQLFHKK
	BPI.34	36	KSKAGWLIQLFHKK
	BPI.35	37	KSKVAWLIQLFHKK
	BPI.36	38	KSKVGALIQLFHKK
15	BPI.37	39	KSKVGWAIQLFHKK
	BPI.38	40	KSKVGWLAQLFHKK
	BPI.39	41	KSKVGWLIQLFHKK
	BPI.40	42	KSKVGWLIQAFHKK
	BPI.41	43	KSKVGWLIQLAHKK
20	BPI.42	44	KSKVGWLIQLFAKK
	BPI.43	45	KSKVGWLIQLFHAK
	BPI.44	46	KSKVGWLIQLFHKA
	BPI.82	76	KSKVGWLIQLWHKK
	BPI.85	79	KSKVLWLIQLFHKK
25	BPI.86	80	KSKVGWLILLFHKK
	BPI.87	81	KSKVGWLIQLFLKK
	BPI.91	86	KSKVGWLIFLFHKK
	BPI.92	87	KSKVGWLIKLFHKK
	BPI.94	89	KSKVGWLIQLFFKK
30	BPI.95	90	KSKVFWLIQLFHKK
	BPI.96	91	KSKVGWLIQLFHKF
	BPI.97	92	KSKVKWLIQLFHKK

TABLE VII (cont'd.)

	<u>BPI Peptide</u>	<u>Seq ID No.</u>	<u>Peptide Sequence</u>
5	IV. Double amino acid substitution peptides		
	Domain II		
	<u>Peptides</u>		
	BPI.45	31	IKISGKWKAAARFLK
10	BPI.56	47	IKISGKWKAKQRFLK
	BPI.59	30	IKISGAWAAQKRFLK
	BPI.60	32	IAISGKWKAQKRFLA
	BPI.88	82	IKISGKWKAFFRFLK
	Domain III		
	<u>Peptides</u>		
15	BPI.100	94	KSKVKWLIKLFHKK
	Va. Double amino acid substitution/combination peptides		
	Domain II		
	<u>Peptides</u>		
20	BPI.46	57	KWKAAARFLKKWKAQKRFLK
	BPI.47	58	KWKAQKRFLKKWKAAARFLK
	BPI.48	59	KWKAAARFLKKWKAAARFLK
	Vb. Multiple amino acid substitution/combination peptides		
	Domain II		
	<u>Peptides</u>		
25	BPI.69	60	KWKAAARFLKKWKAAARFLKKWKAAARFLK
	BPI.99	93	KWKAQWRFLKKWKAQWRFLKKWKAQWRFLK
	BPI.101	95	KSKVKWLIKLFKFKSKVKWLIKLFKFK
	VIa. Atypical amino acid substitution peptides		
	Domain II		
	<u>Peptides</u>		
30	BPI.66	49	IKISGKW _D KAQKRFLK
	BPI.67	50	IKISGKA _{β-(1-naphthyl)} KAQKRFLK
	BPI.70	63	IKISGKWKA _{β-(3-pyridyl)} QKRFLK
35			

TABLE VII (cont'd.)

	BPI Peptide	Seq ID No.	Peptide Sequence
5			
	Vla. Atypical amino acid substitution peptides (cont'd.)		
	Domain II		
	<u>Peptides</u>		
	BPI.71	66	A _D A _D IKISGKWKAQKRFLK
10	BPI.72	64	IKISGKWKAQKRA _β -(3-pyridyl)
	BPI.76	71	IKISGKWKAQF _D RFLK
	BPI.80	74	IKISGKWKAQA _β -(1-naphthyl)RFLK
	Domain III		
	<u>Peptides</u>		
15	BPI.83	77	KSKVGA _β -(1-naphthyl)LIQLFHKK
	Vlb. Atypical amino acid double substitution peptides		
	Domain II		
	<u>Peptides</u>		
	BPI.84	78	IKISGKA _β -(1-naphthyl)KAQFRFLK
20	BPI.89	84	IKISGKA _β -(1-naphthyl)KAFKRFLK
	Vlc. Atypical amino acid triple substitution peptides		
	Domain II		
	<u>Peptides</u>		
	BPI.90	85	IKISGKA _β -(1-naphthyl)KAFFRFLK
25	VII. Cyclized peptides		
	Domain II		
	<u>Peptides</u>		
	BPI.58	9	CIKISGKWKAQKRFLK
30	BPI.65 oxidized	10	CIKISGKWKAQKRFLKC

TABLE VII (cont'd.)

	BPI Peptide	Seq ID No.	Peptide Sequence
5	VII. Cyclized peptides		
	Domain II Peptides		
10	BPI.65 reduced	10	CIKISGKWKAQKRFLKC
	VIIIa. Interdomain combination peptides		
	Domain II- Domain III Peptides		
15	BPI.30	52	KWKAQKRFLKKSKVGWLIQLFHKK
	BPI.63	53	IKISGKWKAQKRFLKKSKVGWLIQLFHKK
	BPI.74	70	KSKVGWLIQLFHKKKKWKAQKRFLK
	VIIIb. Interdomain combination multiple substitution peptides		
	Domain II- Domain III Peptides		
20	BPI.102	96	KWKAQFRFLKKSKVGWLILLFHKK
	VIIIc. Atypical amino acid double substitution/interdomain combination peptides		
	Domain II- Domain III Peptides		
25	BPI.93	88	IKISGKA _{β-(1-naphthyl)} KAQFRFLKKSKVGWLIQLFHKK
	BPI.98	83	IKISGKA _{β-(1-naphthyl)} KAQFRFLKKSKVGWLIFLFHKK
30			

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli 15</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.1	^b	-	-	-	-	-
BPI.2	> 2733.5	> 5	-	-	-	-
BPI.3	696	2.14	-	-	N.T. ^c	N.T.
BPI.4	-	-	-	-	-	-
BPI.5	398	1.05	> 1904	> 5	N.T.	N.T.
BPI.6	-	-	-	-	-	-
BPI.7	175	0.46	> 1890.6	> 5	> 1890.6	> 5
BPI.8	> 3797.1	> 5	-	-	N.T.	N.T.
BPI.9	479	1.02	> 2345.9	> 5	N.T.	N.T.
BPI.10	102	0.41	697	2.76	N.T.	N.T.
BPI.11	638	1.06	-	-	N.T.	N.T.
BPI.12	525	1.78	-	-	N.T.	N.T.

5

10

15

20

TABLE VIII

Bactericidal Activity^a

<u>BPI Peptide</u>	<u><i>E. coli</i> J5</u>		<u><i>E. coli</i> O111:B4</u>		<u><i>S. aureus</i></u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.13	441	0.75	> 2923.9	> 5	> 2923.9	> 5
BPI.14	-	-	-	-	N.T.	N.T.
BPI.15	> 2797.8	> 5	-	-	N.T.	N.T.
BPI.16	> 2821.5	> 5	-	-	N.T.	N.T.
BPI.17	> 2807.2	> 5	-	-	N.T.	N.T.
BPI.18	> 2757.6	> 5	-	-	N.T.	N.T.
BPI.19	> 2712.8	> 5	-	-	N.T.	N.T.
BPI.20	> 2821.5	> 5	-	-	N.T.	N.T.
BPI.21	> 2917	> 5	-	-	N.T.	N.T.
BPI.22	> 2821.50	> 5	-	-	N.T.	N.T.
BPI.23	1330	2.36	> 2821.15	> 5	N.T.	N.T.
BPI.24	655	1.16	> 2821.50	> 5	N.T.	N.T.

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli J5</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.25	> 2866.8	> 5	-	-	N.T.	N.T.
BPI.26	> 2852.1	> 5	-	-	N.T.	N.T.
BPI.27	> 2797.8	> 5	-	-	N.T.	N.T.
BPI.28	> 2821.5	> 5	-	-	N.T.	N.T.
BPI.29	442	1.5	> 1469.2	> 5	> 1469.2	> 5
BPI.30	76	0.23	608	1.84	1216	3.68
BPI.31	938	1.55	-	-	N.T.	N.T.
BPI.32	614	1.04	-	-	N.T.	N.T.
BPI.33	575	0.95	-	-	N.T.	N.T.
BPI.34	916	1.54	-	-	N.T.	N.T.
BPI.35	263	0.45	-	-	N.T.	N.T.
BPI.36	1652	2.64	-	-	N.T.	N.T.

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli J5</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.37	1284	2.14	-	-	N.T.	N.T.
BPI.38	1698	2.83	-	-	N.T.	N.T.
BPI.39	316	0.52	-	-	N.T.	N.T.
BPI.40	1760	2.94	-	-	N.T.	N.T.
BPI.41	2465	4.03	-	-	N.T.	N.T.
BPI.42	265	0.44	> 3041.3	> 5	N.T.	N.T.
BPI.43	729	1.21	> 3024.8	> 5	N.T.	N.T.
BPI.44	481	0.8	2983	4.93	N.T.	N.T.
BPI.45	1302	2.23	> 1696.7	> 5	> 1696.7	> 5
BPI.46	186	0.47	> 1811.2	> 5	> 1811.2	> 5
BPI.47	98	0.25	577	1.46	> 2461.9	> 5
BPI.48	42	0.1	254	0.61	> 1390.4	> 5

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli J5</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.49	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.50	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.51	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.52	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.53	-	-	-	-	N.T.	N.T.
BPI.54	-	-	-	-	N.T.	N.T.
BPI.55	299	0.75	> 1592.2	> 5	> 1592.2	> 5
BPI.56	1387	2.54	-	-	-	-
BPI.57	514	1.05	-	-	-	-
BPI.58	1050	2.03	-	-	-	-
BPI.59	> 2312.3	> 5	-	-	-	-
BPI.60	> 2136.5	> 5	-	-	-	-

TABLE VIII

Bactericidal Activity^a

<u>BPI Peptide</u>	<u>E. coli J5</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μg/well)	(pmol/well)	(μg/well)	(pmol/well)	(μg/well)
BPI.61	> 2093.5	> 5	-	-	-	-
BPI.62	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.63	87	0.31	512	1.8	> 1006.3	> 5
BPI.64	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.65 oxidized	895	1.82	-	-	> 3118	> 5
BPI.65 reduced	1362	2.77	-	-	-	-
BPI.66	> 3496.7	> 5	-	-	-	-
BPI.67	> 1901.8	> 5	-	-	-	-
BPI.68	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.69	57	0.21	244	0.88	1058	3.83
BPI.70	-	-	-	-	-	-

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli</u> <u>15</u>		<u>E. coli</u> <u>O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.71	2297	4.53	-	-	-	-
BPI.72	> 1911.2	> 5	-	-	-	-
BPI.73	57	0.11	> 1810.9	> 5	> 1810.9	> 5
BPI.74	732	2.21	> 2148.2	> 5	> 2148.2	> 5
BPI.75	2030.8	4.96	-	-	> 2030.8	> 5
BPI.76	> 3906.5	> 5	-	-	-	-
BPI.77	455	0.85	-	-	1684.5	3.15
BPI.78	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
BPI.79	> 2282.9	> 5	-	-	-	-
BPI.80	655	1.24	-	-	> 1975.4	> 5
BPI.81	284	0.52	> 2344.9	> 5	> 2344.9	> 5
BPI.82	171	0.32	> 1197.8	> 5	> 1197.8	> 5

5

10

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli J5</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.83	155	0.27	> 2033.5	> 5	> 2033.5	> 5
BPI.84	12	0.02	> 2016.9	> 5	> 2016.9	> 5
BPI.85	227	0.4	> 1881.2	> 5	> 1881.2	> 5
BPI.86	1520	2.58	-	-	> 2048.5	> 5
BPI.87	189	0.32	> 1535.8	> 5	> 1535.8	> 5
BPI.88	70.32	0.13	540.15	1	> 2380.0	> 5
BPI.89	229.09	0.43	> 1882.4	> 5	> 1882.4	> 5
BPI.90	83.11	0.16	1763	3.32	> 1863.3	> 5
BPI.91	> 3843.5	> 5	-	-	-	-
BPI.92	331.8	0.57	-	-	-	-
BPI.93	212.87	0.76	> 980.3	> 5	-	-
BPI.94	922.54	1.59	> 922.5	> 5	> 922.5	> 5

TABLE VIII

Bactericidal Activity^a

<u>BPI</u> <u>Peptide</u>	<u>E. coli J5</u>		<u>E. coli O111:B4</u>		<u>S. aureus</u>	
	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)	(pmol/well)	(μ g/well)
BPI.95	330.88	0.6	> 1397.6	> 5	-	-
BPI.96	378.33	0.65	> 2048.5	> 5	> 2048.5	> 5
BPI.97	296.58	0.53	-	-	-	-
BPI.98	> 1626.1	> 5	> 1626.1	> 5	> 1626.1	> 5
BPI.99	722.9	2.99	> 1064.1	> 5	> 1064.1	> 5
BPI.100	407.74	0.73	> 2655	> 5	-	-
BPI.101	1329.3	4.79	> 1329.3	> 5	> 1329.3	> 5
BPI.102	> 2635.6	> 5	> 2635.6	> 5	-	-
MAP.1	106	0.82	552.79	4.27	> 647.5	> 5
MAP.2	> 690.9	> 5	> 690.9	> 5	> 690.5	> 5

^a Amount added to well to achieve a 30 mm² hold as determined by PROBIT analysis as described in Examples 15 and 16

^b No detectable activity up to 5 μ g/well.

^c N.T. = not tested.

It will be recognized that BPI.84 peptide was found to have bactericidal activity against *E. coli* J5 bacteria that was the molar equivalent to rBPI₂₃.

5 The results of heparin binding experiments for the BPI functional domain peptides of the invention are shown in representative examples in Figures 15a-15e and Figure 16, and summarized in Table IX, wherein heparin binding data are expressed as affinity (nM) and capacity (ng). By plotting the amount of heparin bound versus increasing concentration of heparin, and fitting the data to standardized equations by non-linear least squares methods (GraFit, v.2.0, Erithacus Software, London, England), both the binding constant (K_d) and capacity
10 are calculated.

77

TABLE IX

	<u>BPI Peptide</u>	<u>Heparin Affinity (nM)</u>	<u>Heparin Capacity (ng)</u>
5	BPI.1	no binding	no binding
	BPI.2	346.5	203.6
	BPI.3	780.8	264.5
	BPI.4	335.6	80.8
10	BPI.5	193.4	177.6
	BPI.7	908.0	405.6
	BPI.8	573.8	92.2
	BPI.9	1141.4	212.5
	BPI.10	915.7	548.9
15	BPI.11	743.9	290.5
	BPI.12	284.6	231.5
	BPI.13	984.5	369.1
	BPI.14	396.4	119.3
	BPI.15	315.0	145.4
20	BPI.16	231.0	127.25
	BPI.17	266.5	113.1
	BPI.18	381.2	156.6
	BPI.19	266.5	203.6
	BPI.20	485.1	203.6
25	BPI.21	231.0	50.9
	BPI.22	315.0	135.7
	BPI.23	693.0	285.0
	BPI.24	165.0	427.6
	BPI.25	346.5	107.2
30	BPI.26	231.0	113.1

TABLE IX (cont'd)

	<u>BPI Peptide</u>	Heparin Affinity (nM)	Heparin Capacity (ng)
5	BPI.27	203.8	119.8
	BPI.28	266.5	156.6
	BPI.29	427.4	463.7
	BPI.30	592.2	499.4
10	BPI.31	252.4	205.1
	BPI.32	339.5	217.1
	BPI.33	492.2	230.7
	BPI.34	518.2	205.1
	BPI.35	1083.0	406.0
15	BPI.36	378.7	80.2
	BPI.37	189.3	136.7
	BPI.38	579.1	194.3
	BPI.39	757.3	335.6
	BPI.40	546.9	160.5
20	BPI.41	468.8	119.1
	BPI.42	820.4	369.1
	BPI.43	492.3	283.9
	BPI.44	579.1	335.6
	BPI.45	152.6	160.7
25	BPI.46	1067.0	321.1
	BPI.47	1911.0	576.4
	BPI.48	1415.0	442.3
	BPI.54	237.4	64.3
	BPI.55	367.6	166.1
30	BPI.56	114.6	135.5

TABLE IX (cont'd)

	<u>BPI Peptide</u>	<u>Heparin Affinity (nM)</u>	<u>Heparin Capacity (ng)</u>
5	BPI.58	194.0	231.2
	BPI.59	174.9	106.7
	BPI.60	64.8	120.3
10	BPI.61	58.3	85.2
	BPI.63	599.8	305.1
	BPI.65 (ox.)	159.5	190.6
	BPI.65 (red.)	216.0	279.6
	BPI.66	295.7	111.6
15	BPI.67	107.8	250.4
	BPI.69	967.1	450.8
	BPI.70	145.2	59.2
	BPI.71	75.6	158.9
	BPI.72	145.2	102.8
20	BPI.73	227.2	413.4
	BPI.74	218.1	207.3
	BPI.75	96.0	119.8
	BPI.76	127.9	144.4
	BPI.77	301.9	581.7
25	BPI.79	199.4	110.2
	BPI.80	135.6	210.3
	BPI.81	334.7	318.4
	BPI.82	427.2	163.1
	BPI.83	409.9	253.3
30	BPI.84	1003.2	329.2
	BPI.85	682.4	233.1

TABLE IX (cont'd)

		Heparin Affinity (nM)	Heparin Capacity (ng)
5	<u>BPI Peptide</u>		
	BPI.86	383.1	208.4
	BPI.87	575.0	280.0
	BPI.88	1629.0	352.8
10	BPI.89	1199.4	252.8
	BPI.90	1231.7	274.8
	BPI.91	288.1	181.2
	BPI.92	667.1	227.3
	BPI.93	386.7	291.5
15	BPI.94	406.9	216.1
	BPI.95	551.2	224.5
	BPI.96	468.8	203.8
	BPI.97	765.4	252.2
	BPI.98	683.3	1678.4
20	BPI.99	9097.7	971.4
	BPI.100	2928.9	314.0
	BPI.101	1905.0	210.9
	BPI.102	4607.8	535.2
	MAP.1	936.8	459.1
25	MAP.2	785.5	391.2
	Cecropin	395.3	242.0
	Magainin	3174.6	453.7
	PMB Peptide	309.42	58.01
30	LALF	1294.1	195.3

An intriguing relationship was observed among representative BPI functional domain peptides when a multiple regression analysis was done using bactericidal activity as the predicted variable and heparin binding capacity and affinity (K_d) as the predictor variables. This analysis revealed that only heparin binding capacity was significantly related to bactericidal activity (heparin capacity, $p = 0.0001$ and heparin affinity, $p = 0.6007$). In other words, the amount of heparin that a given peptide embodiment can bind at saturation (*i.e.* capacity) has a significant relationship with bactericidal activity and not how soon a given peptide reaches 50% saturation in the heparin titration (*i.e.* affinity). From the data on LPS binding competition and neutralization, it also appears that capacity is most predictive of bactericidal activity. For examples, the results demonstrate that BPI.7, BPI.29, BPI.30, BPI.46, BPI.47, BPI.48, BPI.63, BPI.65 (reduced), BPI.69, BPI.73, BPI.58, MAP.1 and MAP.2 have extremely high heparin capacity and also are highly bactericidal. Multiple antigenic peptides (MAP peptides) are multimeric peptides on a branching lysine core as described by Posnett and Tam, 1989, *Methods in Enzymology* 178: 739-746. Conversely, BPI.2, BPI.4, BPI.8, BPI.14, BPI.53 and BPI.54 have low heparin binding capacity and accordingly have little or no bactericidal activity.

BPI interdomain combination peptides BPI.30 (comprising domain II-domain III peptides) and BPI.74 (comprising domain III-domain II peptides) were compared for bactericidal activity against Gram-negative and Gram-positive bacteria, and for heparin binding and capacity. These results surprisingly showed that inverting the order of the peptides in the combination changed the relative activity levels observed. For example, BPI.74 was found to have greatly reduced bactericidal activity compared with BPI.30. Specifically, BPI.74 had 10-fold lower bactericidal activity against *E. coli* J5 bacteria, 50-fold lower bactericidal activity against *E. coli* O111:B4 bacteria, and 3.5-fold lower bactericidal activity against *S. aureus*. A 2-fold reduction in heparin binding capacity and a 2-fold increase in heparin affinity, was also observed.

Other bactericidal and endotoxin binding proteins were examined for heparin binding activity. Cecropin A, magainin II amide, Polymyxin B peptide and

Limulus anti-LPS factor (LALF) were assayed in the direct heparin binding assay described in Example 3. The magainin II amide (Sigma, St. Louis, MO) exhibited the highest heparin binding capacity (437.7 ng heparin/2 μ g peptide, $K_d=3.17 \mu$ M) relative to cecropin A (Sigma, 242 ng/2 μ g, $K_d=395$ nM), LALF (Assoc. of Cape Cod, Woods Hole, MA, 195.3 ng/2 μ g peptide, $K_d=1.29 \mu$ M), and PMB peptide (Bachem Biosciences, Philadelphia, PA, 58.0 ng/2 μ g peptide, $K_d=309$ nM). The magainin II amide is a substitution variant of the natural magainin sequence, where 3 alanines have been substituted at positions 8, 13, 15. The magainin II amide is reported to have less hemolytic activity than the natural magainin sequence.

The above results support the relationship between heparin binding, LPS binding and bactericidal activities demonstrated by the BPI peptide data and suggest that other LPS binding proteins will also bind to heparin. The more active bactericidal proteins, cecropin A and magainin II amide, correspondingly, have the highest heparin binding capacity of this series of other LPS binding proteins.

One type of BPI functional domain peptide addition variant incorporates the addition of D-alanine-D-alanine to either the amino- or carboxyl-terminus of a BPI functional domain peptide. The rationale for this approach is to confer greater Gram-positive bactericidal activity with the addition of D-alanine. The cell wall biosynthesis in Gram-positive bacteria involves a transpeptidase reaction that specifically binds and utilizes D-alanine-D-alanine. Beta-lactam antibiotics such as the penicillins effectively inhibit this same reaction. Incorporation of D-alanine-D-alanine onto an active bactericidal peptide should target the peptide to the actively growing cell wall of Gram-positive bacteria.

In the domain II substitution series of BPI functional domain peptides, an unexpected increase was observed when Lys₉₅ was substituted by alanine (BPI.24). A subsequent phenylalanine substitution at position 95 (BPI.73) resulted in improved activity compared with the alanine substitution species. Surprisingly, substitution at position 95 with D-Phe (BPI.76) resulted in dramatically reduced activity, to levels lower than the original peptide (BPI.2). This isomer effect demonstrates that the interactions of this peptide is stereospecific, and implies that BPI.73 can adopt a more active conformation compared with BPI.76. Such

stereospecificity, particularly after the phenomenon has been investigated at other residues, provides an important determinant for pharmacophore development.

Peptides derived from the functional domains of BPI as defined herein have been utilized to determine that the hydrophobic amino acids (especially tryptophan) are most critical for optimal activity. This finding was unexpected due the cationic nature of BPI. In fact, for domain II, when a lysine is replaced by an alanine or phenylalanine, the activity increases dramatically (BPI.24, BPI.73). Combinations of functional domain peptides can also increase the potency of individual peptide constructs, including combinations of the most active substitution peptides from the three domains.

The purity of each newly synthesized peptide was determined by analytical reverse-phase HPLC using a VYDAC C-18 column (25 cm x 4.6 mm, 5 μ m particle size, 30 nm pore size; Separation Group, Hesperia, CA). HPLC was performed using 5% acetonitrile/0.1% trifluoroacetic acid (TFA) in water as mobile phase A, and 80% acetonitrile/0.065% TFA as mobile phase B. The eluate was monitored spectrophotometrically at 220 nm. The flow rate was 1.0 mL/min. Gradient elution conditions were selected to give optimum resolution for each peptide. Purity was expressed as the percentage that the main peak area contributed to the total peak area (see Table X). Purity and identity of the new synthesized peptides were also determined by electrospray ionization mass spectrometry using a VG Biotech Bio-Q mass spectrometer. Table X presents a summary of the purity analyses of exemplary peptides of the invention by mass spectroscopy and HPLC.

84

TABLE X

	Peptide #	Protein AA Segment	MS % Purity	HPLC % Purity
	BPI.1	19-33	-	-
	BPI.2	85-99	57	37.2
5	BPI.3	73-99	-	-
	BPI.4	25-46	-	-
	BPI.5	42-163	-	-
	BPI.6	112-127	-	-
	BPI.7	(90-99) x 2	69	40.9
10	BPI.8	90-99	79	-
	BPI.9	95-99,90-99	-	-
	BPI.10	94-99, 90-99, 90-99 and 93-99, 90-99, 90-99	-	-
	BPI.11	148-151,153-161	-	-
	BPI.12	141-169	-	-
15	BPI.13	148-161	78	69
	BPI.13P	148-161	100	98
	BPI.14	21-50	-	13,3
	BPI.15	85-99, A @ 85 (I)	66	57.6
	BPI.16	85-99, A @ 86 (K)	-	84.1
20	BPI.17	85-99, A @ 87 (I)	86	77,67
	BPI.18	85-99, A @ 88 (S)	66	70
	BPI.19	85-99, A @ 88 (G)	-	69
	BPI.20	85-99, A @ 90 (K)	-	66
	BPI.21	85-99, A @ 91 (W)	68	65.8

TABLE X (cont'd)

	Peptide #	Protein AA Segment	MS % Purity	HPLC % Purity
	BPI.23	85-99, A @ 94 (Q)	-	69
	BPI.24	85-99, A @ 95 (K)	-	67
5	BPI.25	85-99, A @ 96 (R)	-	73
	BPI.26	85-99, A @ 97 (F)	-	73
	BPI.27	85-99, A @ 98 (L)	-	65
	BPI.28	85-99, A @ 99 (K)	-	80
	BPI.29	(148-161) x 2	-	-
10	BPI.30	90-99,148-161	-	21
	BPI.30-P	90-99,148-161	95	98
	BPI.31	148-161, A @ 148 (K)	-	68
	BPI.32	148-161, A @ 149 (S)	-	70
	BPI.33	148-161, A @ 150 (K)	-	58
15	BPI.34	148-161, A @ 151 (V)	-	51
	BPI.35	148-161, A @ 152 (G)	-	72
	BPI.36	148-161, A @ 153 (W)	-	64
	BPI.37	148-161, A @ 154 (L)	-	51
	BPI.38	148-161, A @ 155 (I)	-	70
20	BPI.39	148-161, A @ 156 (Q)	-	53
	BPI.40	148-161, A @ 157 (L)	-	53
	BPI.41	148-161, A @ 158 (F)	-	63
	BPI.42	148-161, A @ 159 (H)	-	59
	BPI.43	148-161, A @ 160 (K)	-	53
25	BPI.44	148-161, A @ 161 (K)	-	70
	BPI.45	85-99, A @ 94(Q)&95(K)	71	46
	BPI.46	(99-90)x2, A @ 1st 94(Q)&95(K)	67	47

TABLE X (cont'd)

	Peptide #	Protein AA Segment	MS % Purity	HPLC % Purity
	BPI.47	(90-99)x2, A @ 2d 94(Q)&95(K)	57	34
	BPI.48	(90-99)x2, A @ both 94(Q)&95(K)	68	33
5	BPI.54	21-35	-	-
	BPI.55	152-172	-	-
	BPI.56	85-99, K @ 94 (Q) & Q @ 95(K)	-	55
	BPI.58	Cys-85-99	49	25.7
	BPI.59	85-99, A @ 90(K)&92(K)	56	30.3
10	BPI.60	85-99, A @ 86(K)&99(K)	57	78.3
	BPI.61	85-99, F @ 91(W)	60	59.8
	BPI.63	85-99, 148-161	38	31.3
	BPI.65 Rd	Cys-85-99-Cys	41	22, 34
	BPI.65 Ox	Cys-85-99-Cys	-	-
15	BPI.66	85-99, W _D @ 91(W)	-	-
	BPI.67	85-99, β -(1-naphthyl)-A @ 91	65	52
	BPI.69	[90-99, A @ 94 (Q) & 95 (K)] x 3	44	54, 40
	BPI.70	85-99, β -(3-pyridyl)-A @ 91	66	54
	BPI.71	A _D -A _D -85-99	-	60
20	BPI.72	85-99, β -(3-pyridyl)-A @ 97 (F)	-	52
	BPI.73	85-99, F @ 95 (K)	-	44, 39
	BPI.74	148-161, 90-99	-	29
	BPI.75	KKRAISFLGKKWQK	-	32
	BPI.76	85-99, F _D @ 95 (K)	-	39

TABLE X (cont'd)

	Peptide #	Protein AA Segment	MS % Purity	HPLC % Purity
	BPI.77	85-99, W @ 95 (K)	-	38
	BPI.79	85-99, K @ 94 (Q)	-	48
5	BPI.80	85-99, β -(1-naphthyl)-A @ 95 (K)	-	44
	BPI.81	85-99, F @ 94 (Q)	-	33, 35
	BPI.82	148-161, W @ 158 (F)	-	58
	BPI.83	148-161, β -(1-naphthyl)-A @ 153 (W)	-	63
	BPI.84	85-99, β -(1-naphthyl) A @ 91 (W) & F @ 95 (K)	-	50
10	BPI.85	148-161, L @ 152 (G)	-	74
	BPI.86	148-161, L @ 156 (Q)	-	51
	BPI.87	148-161, L @ 159 (H)	-	63
	BPI.88	85-99, F @ 94 (Q) & 95 (K)	-	50
	BPI.89	85-99, β -(1-naphthyl) A @ 91 (W) & F @ 94 (Q)	-	50
15	BPI.90	85-99, β -(1-naphthyl) A @ 91 (W), F @ 94 (Q) & 95 (K)	-	63
	BPI.91	148-161, F @ 156 (Q)	-	31
	BPI.92	148-161, K @ 156 (Q)	-	50
	BPI.93	85-99 148-161 β -(1-naphthyl) A @ 91 (W), F @ 95 (K)	-	38
	BPI.94	148-161, F @ 159 (H)	-	59
20	BPI.95	148-161, F @ 152 (G)	-	57
	BPI.96	148-161, F @ 161 (K)	-	60

88

TABLE X (cont'd)

	Peptide #	Protein AA Segment	MS % Purity	HPLC % Purity
	BPI.97	148-161, K @ 161 (G)	-	67
	BPI.98	90-99, β -(1-naphthyl) A @ 91 (W), F @ 95 (K) + 91	-	31
5	BPI.99	[90-99, W @ 95 (K)]x3	-	-
	BPI.100	148-161, K @ 152 (G) & 156 (Q)	-	-
	MAP.1	β Ala-N α ,N ϵ -[N α ,N ϵ (BPI.2)1Lys]Lys	54	multiple peaks
	MAP.2	β Ala-N α ,N ϵ -[N α ,N ϵ (BPI.13)1Lys]Lys	49	multiple peaks

10